UNIVERSITÀ
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Administrative unit: **Università degli Studi di Padova**

Department: **Territorio e Sistemi Agro-Forestali (TESAF)**

PhD Program: **Land, Environment, Resources and Health (LERH)**
Batch: **XXX**

Dendroanatomy: a new approach to sharpen the focus on the climatic drivers of tree growth in the Mediterranean.

PhD Program Coordinator: Prof. Davide Matteo Pettenella

Supervisor: Prof. Marco Carrer

Co-Supervisor: Dr. Jesús Julio Camarero, IPE, Zaragoza, Spain

External evaluators:

Dr.Paolo Cherubini, WSL, Birmensdorf, Switzerland

Prof. Veronica De Micco, University of Naples Federico II, Italy

PhD candidate: **Arturo Pacheco Solana**

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“The clearest way into the Universe

is through a forest wilderness.”

— John Muir

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Summary

Climate change is the biggest challenge of our time and the Mediterranean basin one of the hot spots where its effects are already tangible. For this region most climate models predict higher temperatures and diminishing rainfall with likely longer and more frequent drought events which represent the principal threat for tree species, affecting growth, mortality and composition of future forest.

Using cutting edge techniques for sample preparation and microscopic image analysis, this dissertation assessed the growth responses of xylem anatomy to climatic constraints. This dendroanatomical approach permits the long-term retrospective analysis of intra-annual growth information permanently fixed and archived in secondary xylem tissue. Wood anatomical results were contrasted and complemented by the use of many other methodologies like xylogenesis, dendrometers and non-structural carbohydrate analysis.

The main species targeted was Aleppo pine (*Pinus halepensis*). Its growth pattern was firstly compared with another coexisting conifer (*Juniperus thurifera*) and their correlations with climate variables evaluated. Afterwards, the bimodal growth pattern shown by this species was assessed in two distinct climatic zones (continental *vs*. oceanic). Finally, three groups of trees under different irrigation treatments were compared. The dendroanatomical analysis of all three stages comprised always a time span of about 40 years, while the complementary methodologies varied according to the different experimental settings.

Applying this dendroanatomical approach it was possible to link xylogenesis with wood anatomy on a decadal-long time scale and explain the climatic correlations that drive the bimodal growth patterns commonly reported in Mediterranean species. In addition, it was possible to disentangle the climatic cues influencing the growth plasticity of Aleppo pines under different conditions: where climate is milder the bimodality in growth is stronger. At the end, the irrigation trial highlighted that growth performances and xylem anatomy can be significantly affected by water availability and that trees tend to maintain a fairly good coordination between C supply and demand even during prolonged drought.

This dissertation improves the general understanding of how higher temperatures and drier conditions will impact and influence the growth and distribution of the Aleppo pine. As one of the most common tree species of the Mediterranean region, the results of these studies can help to understand and predict the future forest dynamics under an uncertain climatic future.

Sommario

Il cambiamento climatico è la più grande sfida dei nostri tempi e i suoi effetti sono già tangibili in varie aree come ad esempio il bacino del Mediterraneo. La maggior parte dei modelli climatici prevedono, per questa regione, un contemporaneo innalzamento delle temperature e diminuzione delle precipitazioni con conseguente aumento di durata e frequenza degli eventi siccitosi. Tale situazione rappresenterebbe la principale minaccia per le specie arboree influenzandone la crescita, la mortalità e modificandone la composizione delle future foreste.

Grazie all'utilizzo di tecniche all'avanguardia nella preparazione dei campioni e analisi delle immagini al microscopio, questa tesi ha individuato le risposte anatomiche all'accrescimento in condizioni climatiche limitanti. Questo approccio dendroanatomico consente l'analisi retrospettiva a lungo termine delle informazioni di accrescimento intra-annuale permanentemente impresse e cronologicamente archiviate nello xilema. I risultati dell'analisi anatomica sono stati comparati e integrati dall'utilizzo di altre metodiche che comprendevano l'analisi della xilogenesi e dei carboidrati non strutturali e l'utilizzo di dendrometri.

Il pino d'Aleppo (*Pinus halepensis*) è la principale specie considerata in questo studio. Inizialmente, si sono valutate le correlazioni con le variabili climatiche e le dinamiche di accrescimento per questa specie rispetto a un'altra conifera compresente (*Juniperus thurifera*). Successivamente, le dinamiche bimodali di accrescimento evidenziate nel pino sono state analizzate in due distinte zone climatiche (continentale e oceanica). Infine, sono stati confrontati tre gruppi di pini d'Aleppo sottoposti a diversi trattamenti di irrigazione. In tutte e tre le fasi precedenti, l'analisi dendroanatomica ha sempre considerato un intervallo temporale di circa 40 anni, mentre si sono adottate diverse tecniche di analisi in relazione alle specifiche impostazioni sperimentali.

Applicando questo approccio dendroanatomico è stato possibile collegare la xilogenesi con l'anatomia del legno su una scala decennale e quindi spiegare le correlazioni climatiche che guidano i modelli bimodali di crescita nella regione mediterranea. Inoltre, è stato possibile individuare i fattori climatici che influenzano la plasticità xilematica a lungo termine e di conseguenza l'accrescimento dei pini d'Aleppo in diverse condizioni climatiche: dove il clima risulta più mite la bimodalità nella crescita è più evidente. Infine, l'esperimento di irrigazione controllata ha evidenziato come la crescita e l'anatomia del legno possano essere influenzate in modo significativo dalla disponibilità idrica e come le specie studiate tendano a mantenere un buon coordinamento tra domanda e disponibilità di carbonio anche durante periodi di siccità prolungata.

Questa tesi contribuisce alla comprensione di come, in un futuro climatico incerto, con l’innalzamento delle temperature e l’aumento degli eventi siccitosi verrà influenzata la crescita e la distribuzione del pino Aleppo, e delle estese foreste dominate da questa specie nella regione mediterranea.

1. General Introduction

1.1 Research background and justification

Long-term climate effects on tree growth has been studied for many years from a broad perspective using classical dendrochronology considering just tree-ring width. This approach can disclose the effects of climate over stem growth on a yearly resolution. Intra-annual climatic signals are recorded with better accuracy by cell development processes. New technical advances allow nowadays the measurement of wood anatomy features like lumen area and cell wall thickness on large amounts of cells, permitting a higher resolution analysis of the effects of weather events on the growth of trees. One of these new tools is Roxas (von Arx and Carrer 2014), a software for automated image analysis on wood samples. Collaboration between the developer of this software and our laboratory has resulted on a deep understanding of the best protocols for sample preparation in order to take advantage of the full potential of this software. The ability of doing dendro-research on a cell by cell wood anatomy level is opening a huge window for studying the relationships between tree growth, the environment changes and even the management decisions on forest plantations. For example, intra annual density fluctuations (IADFs) are a common result of dry periods during the trees growing season. Understanding how climate variability drive the formation of IADFs, will help managing the forests under the future different predicted scenarios of climate change, especially for Mediterranean and dry tropical forest areas subjected to drought and higher temperatures.

1.2 State of knowledge on the research topic

Dendroanatomy is a new terminology that has been proposed to describe the new approaches on dendrochronology where wood anatomical traits are analysed along series of dated tree rings. Lumen area and cell wall thickness measured through time (at the intra- and inter-annual level) can characterize the relationships between tree growth and various environmental factors. There is a lack of studies at the intra-specific level regarding the long-term modifications of xylem over the full life-span of trees, and its variability along axial and radial profiles (Streit et al., 2014). The study of dendroanatomy is emerging as a promising approach in tree biology and climate change research, particularly if complemented by physiological and ecological studies (Fonti et al., 2010).

Tree-ring variables such as ring width or maximum latewood density have been shown to be strongly influenced by environmental conditions, especially where temperature or precipitation limits tree growth. (Marshall, 2014). There are other widely studied characteristics of wood, for example its anatomical structure, which can encode additional and new ecological information (Fonti et al., 2010). Application of dendrochronological techniques to wood anatomy have opened new frontier to better characterize the relationship between tree growth and various environmental factors in time, mainly increasing the time resolution. The growing number of papers published in the recent years confirms the promising potential of this approach. (Fonti et al., 2009; Olano et al., 2013).

Variation in wood anatomical characteristics represents adaptive structural solutions adopted by the tree in order to achieve an optimal balance among the competing needs of support, storage and transport under changing environmental conditions and phylogenetic constraints (Change & Paper, 2007). Studies of variations in xylem anatomy have already been an important source of information in plant sciences (Gartner et al., 2000).

Until recently, wood anatomists have advanced the understanding of phylogenetic adaptations in plants by analysing and interpreting variation of wood structures across taxa and climatic zones, intraspecific variation across climatic zones, along environmental gradients, or between contrasting sites supplying additional information about the linkage between ecology (habitat) and functioning (derived from xylem anatomy) (Wimmer, 2002).

The close relationship between climate and plants is reflected in functional adaptations and large morphological and taxonomic diversity. One important functional trait represents water transport capacity through the xylem tissue, which is closely connected with a plant’s water use strategy and net primary productivity in a given environment. In fact, the xylem structure reflects the functional balance between efficient water transport to achieve optimal growth, minimum investment of construction costs to secure the xylem hydraulic system, mechanical support of the assimilating leaves, and storage of water and non-structural carbohydrates for defence and resistance to stress (von Arx et al., 2012).

Although water-conducting cells are dead when fully developed and functional, woody plants are able to acclimate their developing xylem to the changing environment before cell death, which is particularly relevant in perennial plants with a long generation cycle. Since these structural features remain permanently fixed and chronologically archived in wood, tree-ring anatomy offers the opportunity to add a ‘time component’ to functional mechanisms of xylem plasticity, and thus to reconstruct dynamics in ecological conditions (Fonti et al., 2010), Its variability across tree rings can thus provide a retrospective of plant's hydraulic and functional adjustments (Bryukhanova & Fonti, 2012).

Improving the knowledge in xylem plasticity is important to link the climate changes with the intra-annual tree growth and to explore limitations and future challenges in how wood density may respond to climate. It is also important to understand how plants balance their hydraulic architecture to optimize growth and minimize costs together with the water use required for the integration of structure functionality of the xylem at various scales.

To date, cell anatomical analyses along tree rings have explored the potential for obtaining high-resolution proxies through identification of the environmental factor(s) and the period of the growing season that primarily influences year to year cell lumen variability. Analyses of inter-annual variability in the size of water conducting cells - vessels in deciduous trees and tracheids in conifers - have shown that conduits can carry sub-annual information reflecting changes in regional environmental conditions (Fonti et al., 2010). The study of these changes in relation to the climatic variability can likely provide valuable insight of tree acclimation.

The Earth's climate has warmed by approximately 0.6 ºC over the past 100 years (Stocker et al., 2013). By looking with a perspective of the recent global warming (Fehr & Fischbacher, 2004), understanding past climate and present trends is becoming more and more important, especially considering regions like the Mediterranean basin. For these area it is expected warmer and drier conditions over the late 21st century (Ipcc, 2013). This trend to aridification has been predicted to be particularly intense during springtime when radial growth rates of Mediterranean evergreen conifers are in their highest, and therefore these conditions would negatively affect the productivity of the tree species that dominate these dry and disturbed areas (Sarris et al., 2007). In the case of Mediterranean areas subjected to harder continental conditions, conifer growth is constrained on two fronts: winter low temperatures and summer drought (Granda et al., 2014). Considering this double seasonal climatic stress, summer and winter conditions can show opposite effects on tree growth and wood anatomy (Andreu et al., 2007; Martin-Benito et al., 2013; Gea-Izquierdo et al., 2014). This means that warm or wet winter and spring can enhance carbohydrate synthesis and growth, whereas hot and dry summer can constrain growth by shortening the growing season (Pasho et al., 2011; Gimeno et al., 2012). This peculiar behaviour lead us to think if trees on other regions with similar weather conditions also present this bimodal growth pattern described for some Mediterranean evergreen conifers, if it varies between years and how this affects wood anatomy, tree functioning (e.g., changes in hydraulic conductivity or in cell-wall deposition and wood lignification) and carbon uptake (Camarero et al., 2010; Klein et al., 2013).

One of the most characteristic features on wood anatomy, for samples coming from areas with contrasting water availability conditions throughout the year, are the intra annual density fluctuations (IADFs) (Fritts 2001, Cherubini 2003, De Micco et al. 2014). In the Mediterranean region, these changes on wood density are a common result of dry periods during the growing season or of sudden increase in water availability at the end of the growing season. Understanding how climate variations drive the formation of IADFs, will help managing the forests under the future different predicted scenarios of climate change, especially for Mediterranean and dry tropical forest areas subjected to drought and higher temperatures.

The following studies were performed relaying in wood anatomy analysis as a main tool but at the same time complementing it with many other approaches of tree growth monitoring. Classical dendrochronology, dendrometers measurements, non-structural carbohydrates analyses were also used in combination with daily-resolved weather records to draw a clear image of the growth of conifers in Mediterranean climate and the different strategies adopted to cope with a dry environment that is predicted to become more extreme in the near future.

1.3 Aims of the study

This thesis focuses in the effect of climate on the inter and intra-annual growth dynamics of common conifers on the Mediterranean basin. With an in-depth analysis of the typical bimodal growth pattern, featured by many tree species growing in this region, is possible to disentangle the internal mechanisms these plants develop to cope with the particular challenges posed by Mediterranean climate.

Each one of the studies that pertains to this thesis targeted different aims, which correlate to each other along the research line they all describe.

- **First study:**
    - To characterize how climate influences growth and wood anatomy in two coexisting conifer species.
    - To evaluate how the seasonal changes drive the formation of IADFs.
- **Second study:**
    - To describe and compare the intra-annual patterns of stem radial growth captured by dendrometers and wood anatomy.

    - To assess whether the climatic signal in anatomical traits mirrors the bimodal growth patter recorded by dendrometers.
    - To Determine which are the climatic conditions that trigger the formation of IADFs.
- **Third Study:**
    - To describe and quantifies the effects of artificial irrigation on wood anatomical traits and analyse its relation with the non-structural carbohydrates dynamics.
    - To elucidates which are the long-term effect of irrigation in Aleppo pine xylem anatomy and how the carbon balance is affected by decade-long changes on the water availability.

1.4 Structure of the thesis

This thesis consists on a collection of 3 scientific articles (chapters 2,3 and 4) produced as part of my doctorate work during the last three years.

The first paper entitled *Linking wood anatomy and xylogenesis allows pinpointing climate and drought influences on growth of coexisting conifers in continental mediterranean climate* was published in *Tree Physiology* in 2016. This work describes the differences of growth from a wood anatomy point of view of two conifer species (*Pinus halepensis* and *Juniperus thurifera*) co-existing on a dry continental Mediterranean climate.

The second paper entitled *Disentangling the climate-driven bimodal growth pattern in coastal and continental Mediterranean pine stands* has been accepted in 2017 and is now online in *Science of the total environment*. In this article, dendrometers monitoring and wood anatomical traits chronologies were compared to assess the bimodal growth pattern of Aleppo pine in two distinct Mediterranean climate regimes. Analysis of the differences in IADF formation helped to comprehend how the same species cope with extreme conditions on different sites.

The third and last paper under the title *Drought and irrigation in Aleppo pine: effects on tree-ring growth, xylem anatomical traits and non-structural carbohydrate concentrations after a 40-year long trial,* has been submitted to *Tree Physiology*. For this last research, we focus on quantifying the effects of irrigation on tree-ring growth, wood anatomical traits and analyse the relation with the non-structural carbohydrate dynamics.

2. Linking wood anatomy and xylogenesis allows pinpointing climate and drought influences on growth of coexisting conifers in continental mediterranean climate

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KEYWORDS: intra-annual density fluctuation, *Juniperus thurifera*, *Pinus halepensis*, quantitative wood anatomy, radial growth, xylem.

2.1 Abstract

Forecasted warmer and drier conditions will probably lead to reduced growth rates and decreased carbon fixation in long-term woody pools in drought-prone areas. We therefore need a better understanding on how climate stressors such as drought constrain wood formation and drive changes in wood anatomy. Drying trends could lead to reduced growth if they are more intense in spring, when radial growth rates of conifers in continental Mediterranean climates peak. Since tree species from the aforementioned areas have to endure dry summers and also cold winters, we chose two coexisting species: Aleppo pine and Spanish juniper (10 randomly selected trees per species), to analyze how growth (tree-ring width) and wood anatomical traits (lumen transversal area, cell-wall thickness, presence of intra-annual density fluctuations – IADF− in the latewood) responded to climatic variables (minimum and maximum temperatures, precipitation, soil moisture deficit) calculated for different time intervals. Tree-ring width and mean lumen area showed similar year-to-year variability, which indicates they encoded similar climatic signals. Wet and cool late-winter to early-spring conditions increased lumen area expansion, particularly in pine. In juniper, cell-wall thickness increased when early summer conditions became drier and the frequency of latewood IADFs increased in parallel with late-summer to early-autumn wet conditions. Thus, latewood IADFs of the juniper capture increased water availability during the late growing season which is reflected in larger tracheid lumens. Soil water availability was one of the main drivers of wood formation and radial growth for the two species. These analyses allow long-term (several decades) growth and wood anatomical responses to climate, to be inferred at intra-annual scales which agree with the growing patterns already described by xylogenesis approaches for the same species. A plastic bimodal growth behaviour, driven by dry summer conditions, is coherent with the presented wood-anatomical data. The different wood-anatomical responses to drought stress are observed as IADFs with contrasting characteristics and responses to climate. These different responses suggest distinct capacities to access soil water between the two conifer species.

2.2 Introduction

Anthropogenic climate warming represents a global challenge for forest productivity in drought-prone areas (Reichstein et al. 2013). Models predict a worldwide increase of mean air temperature of ca. 2–4 °C with significant drying over regions such as the Mediterranean Basin (IPCC 2013). Climate warming is expected to magnify drought stress in that area by raising air temperatures and evapotranspiration rates, thus increasing the frequency and severity of droughts and heat waves (Meehl and Tebaldi 2004). Arid and hot conditions could be more intense during spring, when radial growth rates of most Mediterranean conifers peak (Camarero et al. 2010, Vicente-Serrano et al. 2010), with negative consequences on forest growth (Sarris et al. 2007), and related ecosystem services such as carbon fixation in long-lasting wood pools. In addition to summer drought, low winter temperatures also constrain forest growth in continental Mediterranean areas (Gimeno et al. 2012, Granda et al. 2014). Several conifer species respond to these dual stressors with a bimodal radial-growth pattern, i.e. with higher wood-formation rates in periods with optimal climatic conditions, namely spring and autumn (Camarero et al. 2010). This double seasonal climatic stress also implies that spring and autumn climatic conditions can have contrasting effects (formation of earlywood-tracheids at the final part of the ring) on growth and wood-anatomical traits (e.g. tracheid lumen area and cell-wall thickness) (De Luis et al. 2007, Olano et al. 2012, Pasho et al. 2012). In fact, the formation of intra-annual density fluctuations (IADF) in Mediterranean conifers could be related with this bimodal pattern of growth. For instance, latewood IADFs are produced when climate induces the formation of earlywood-like tracheids with wide lumen areas and thin cell walls in summer or autumn when typically thick-wall tracheids with narrow lumen are formed (Campelo et al. 2007). Despite IADFs having been climatically characterized in many Mediterranean conifer species (Vieira et al. 2010, Novak et al. 2013), we still lack a more mechanistic approach to determine how their formation and anatomy respond to climate. A deeper understanding of the processes forming the IADFs justified them as a valuable wood-anatomical feature to be studied to link climate, xylogenesis and wood anatomy as advocated in previous research (Vaganov et al. 2006). Since the bimodal growth pattern has been described following cambium phenology over the course of a few years (cf. Camarero et al. 2010), longer records of IADF production and wood anatomy could identify if that pattern is reflected in the responses of xylem traits to climate variability. Specifically, it could be investigated if lumen area or cell-wall thickness respond differently to seasonally varying climatic stress.

The long-term climate effects on radial growth of conifers are usually analyzed by measuring annually or seasonally-resolved variables such as tree-ring, earlywood or latewood widths using dendrochronology (Fritts 2001). However, quantitative wood anatomy allows information to be obtained on tree functioning (e.g., carbon uptake, water use) and growth to be encoded at much finer temporal scales (Fonti et al. 2010, von Arx and Carrer 2014). Here, we aim to characterize how climate influences growth and wood anatomy in two coexisting conifer species (Spanish juniper and Aleppo pine) subjected to continental Mediterranean conditions but presenting different phenological (e.g., xylogenesis), morphological (e.g., root depth) and physiological (e.g., drought tolerance) features (Gauquelin et al. 1999, Ne'eman and Trabaud 2000, Baquedano and Castillo 2007, Camarero et al. 2010, De Soto et al. 2011). To achieve this, we quantify wood-anatomical traits (lumen area, cell-wall thickness) and relate them to climate variables (temperature, precipitation, water balance, vapour pressure deficit, soil moisture) by contrasting correlation analyses results and xylogenesis data available for both species. We also analyze how climate drives seasonal changes in wood anatomy by evaluating the formation of IADFs in response to specific climatic conditions. We hypothesize that the bimodal pattern of growth is idiosyncratically expressed by the two coexisting conifer species which should show different growth and wood-anatomy responses to climate. We discuss how these different wood responses to climate relate to contrasting species-specific traits, mainly those related to the root depth and the ability of trees to access soil water.

2.3 Materials and methods

2.3.1 Study site and species

We selected a mixed Mediterranean forest (Vedado de Peñaflor) located close to Zaragoza city (41º 47' N, 0º 43' W, 560 m a.s.l.), in the Middle Ebro Basin, northeastern Spain. Based on climate data for the period 1970 to2013 (see below), the climate at the site is continental Mediterranean with an average yearly rainfall of 313 mm (semi-arid conditions), of which 66 mm were recorded between June and August. Annual mean temperature was 13.8 ºC with frosts occurring frequently during winter (Online Resource 1, Appendix S1). The estimated annual water balance was -476 mm with water deficit occurring from March to October. No significant trend was observed in precipitation data of the study area since 1970, but mean maximum temperatures have been steadily rising leading to drier conditions (Appendix S1). Gypsum and marls comprise the parent rock material, while the topography of the terrain consists in small hills and valleys with west and east-facing slopes. Two co-occurring evergreen conifer species belonging to different families were selected: Spanish juniper (*Juniperus thurifera* L, Cupressaseae) and Aleppo pine (*Pinus halepensis* Mill.,Pinaceae). Spanish juniper is endemic to the Western Mediterranean basin, being most abundant in Spain and Morocco, and it usually grows under continental Mediterranean climate conditions on poor or rocky soils (Gauquelin et al. 1999). Spanish Juniper growth is enhanced by wet winter and warm spring conditions, whereas summer water deficit is associated to low growth rates (De Soto et al. 2012). Aleppo pine is the most widely distributed Mediterranean pine species and is well adapted to growth under xeric conditions by tolerating water shortage (Ne'eman and Trabaud 2000). In the study area, Aleppo pine growth is mainly enhanced by wet and cool conditions during spring and in the winter prior to tree-ring formation (Pasho et al. 2012). At the studied site, *J. thurifera* forms a relict population mostly located on valley bottoms and west-facing slopes, and is intermingled with *P. halepensis* which is mostly present on east-facing slopes where xeric conditions are stronger. More details on the study site can be found in Camarero et al. (2010).

2.3.2 Climate data

Daily climatic records were obtained from the Peñaflor-Aula Dei (41° 45' N, 0° 49' W, 280 m) weather station located at 5 km from the study site. In order to fill some missing temperature data gaps from Peñaflor we used the Zaragoza-airport (41° 40' N, 1° 00' W, 263 m) weather station located at 30 km from the study site. We considered the daily-resolved records for mean, maximum and minimum temperatures, relative humidity, and total precipitation for the period 1970-2013. Vapour pressure deficit (VPD) was estimated using temperature and humidity data (Abtew and Melesse 2013). We also calculated the water balance or difference between precipitation and potential evapotranspiration (PET) following (Hargreaves et al. 1982), and the soil moisture deficit (SMD) was obtained as the difference between cumulative evapotranspiration and cumulative rainfall (Cocozza et al. 2012). Daily data were also averaged (temperatures, VPD) or summed (precipitation, water balance) at various time intervals (5, 7, 10, and 15 days) to analyze climate-growth relationships at multiple temporal scales.

2.3.3 Dendrometer and xylogenesis data

To characterize the phenology of radial growth and xylogenesis (phases of xylem formation) we used data of radial stem changes based on manual band dendrometer records (Agricultural Electronics, Tucson, USA) and observations of xylem formation (xylogenesis) based on repeated wood sampling (micro cores), respectively. These data were recorded biweekly or monthly in 10 trees per species. Part of the dendrometer data (2006-2012 period) and all xylogenesis data (years 2006 and 2010) have been already published (Camarero et al. 2010, Pasho et al. 2012). Dendrometer data were used to determine the times of maximum increment and decrement rates that were defined when more than 50% of trees showed positive or negative increment rates, respectively. Xylogenesis allowed the main phases of xylem formation to be characterized in both species by counting different cell developmental phases (cambium cells, radially-enlarging tracheids indicating cambium resumption, thickening tracheids indicating the onset of wall lignification, and mature tracheids (cf. (Antonova and Stasova 1993, Cuny et al. 2014)). More details on these data can be found in Camarero et al. (2010) and Pasho et al. (2012).

2.3.4 Wood-anatomy data acquisition and analyses

In January 2014, a total of 20 dominant trees, between 40-60 years old, (10 per species) were randomly selected and sampled with a Pressler increment borer taking three radial cores per tree at 1-1.3 m. During sampling, we avoided damaged stem areas with visible scars or showing signs of recent injuries and forming compression wood. In the laboratory, two cores per tree were prepared following standard dendrochronological methods (fixed in woody mounts, glued and sanded). These samples were visually cross-dated and then measured to a precision of 0.01 mm using a Lintab measuring system (F. Rinn, Heidelberg, Germany). Cross-dating was statistically validated using the COFECHA program (Holmes 1983). For histological analyses we selected the five best cross-dated trees cores per species, that is trees with cores showing the highest correlation with the mean ring-width series of each tree species. We also considered cores without visible anomalies (e.g. reaction wood, resin pockets) and containing the pith in order to obtain complete tree-ring sequences. Samples were processed following (Gärtner and Schweingruber 2013). Cores were cut in small pieces (3-5 cm long), boiled in water to soften the wood and then cut in thin slices (10 to 20 µm thick) using a rotary microtome. Slices were then cleaned, stained with a safranin (1%) and astra blue (0.5%) (both in destilled water), rinsed with water and alcohol and finally permanently fixed with a synthetic resin (Eukitt™; Merck, Darmstadt, Germany).

Image acquisition was performed with a digital camera mounted on a light microscope; images were captured at 100X magnification. Overlapping images were taken from each sample and stitched together using the PTGui software (New House Internet Services, Rotterdam, NL) to obtain high-resolution images (0.833 pixels µm-1) of the entire section. Tree-ring borders were manually drawn on the images that were semi-automatically analyzed using the ROXAS software (von Arx and Carrer 2014).

We measured the following wood-anatomical traits in the transversal xylem sections: mean lumen area (LA) and radial cell-wall thickness (CWT). The radial dimension of CWT was chosen because it has a higher year-to-year variability than the tangential dimension (Vysotskaya and Vaganov 1989). Then, following Heres et al. (2014), we calculated several parameters related to the hydraulic performance of the tree: i) mean hydraulic diameter (dh); ii) mean percentage conductive area, and iii) theoretical hydraulic conductivity (Kh) computed according to the Hagen-Poiseuille law (Tyree and Zimmermann 2002). The dh was calculated by assuming that lumen areas are circular and using the formula $dh = \Sigma d5/\Sigma d4$, where d is the tracheid diameter (Pockman and Sperry 1997).

To better define the time windows over the phases of radial growth we created 10 chronologies out of LA and CWT based on the relative radial position of each tracheid along the tree ring. This was done by dividing each tree ring in 10 sectors (deciles) along the radial direction and then computing the median LA and CWT values for each decile (see Online Resource 2, Fig. S2). We decided to use deciles because they provided a good trade-off between sample size (number of cells per sector) and spatial and temporal resolution.

To assess the presence of latewood IADFs and calculate their relative frequency (%), we graphed the LA and CWT mean values along the tree-ring for each IADF and compared them with the Mork index, which is commonly used to define the latewood presence (Denne 1988). In this case, the Mork index proved to have a low efficiency in accurately defining earlywood and latewood limits due to the presence of latewood IADFs (see Online Resource 3, Fig. S3). IADFs exclusively found within the latewood were therefore identified visually.

Lastly, we built chronologies of tree-ring width and wood-anatomical variables for each tree species using the ARSTAN program which was specifically developed for the removal of biologically induced age-size related growth trends (Cook 1985), tendencies that are also present in wood-anatomical data (Carrer et al. 2015). Individual series were fitted using a cubic smoothing splines function with 50% frequency–response cutoff of 50 years. Afterwards, observed values were divided by expected values to obtain detrended and dimensionless indices, which were then subjected to autoregressive modelling to remove part of the temporal autocorrelation. These indices were averaged to create the mean chronology of each variable for the two species.

The climate-growth associations were quantified by calculating Pearson correlations between the detrended growth and wood-anatomical chronologies and the different time windows of climatic data for the best-replicated period (1975-2013). In the case of IADF frequencies, we related them with climatic variables using the Spearman non-parametric correlation coefficient (rs). The calculations were computed from previous October to current September based on a previous study (Camarero et al. 2010).

2.4 Results

2.4.1 Wood anatomical features

The quantitative description of wood anatomy was based on rings formed between 1970 and 2013 and included the measurement of $3.0\text{x}10^5$and $4.6 \text{ x } 10^5$ tracheids in Aleppo pine and Spanish juniper, respectively. The two species significantly differ in the distributions of LA and CWT (Fig. 1), with pine typically forming tracheids with larger lumens (pine 276 μm^2 vs. juniper 147 μm^2) and thicker walls (pine 4.1 µm vs. juniper 3.4 µm) (Table 1). Consequently, both the dh and Kh were higher in Aleppo pine than in Spanish juniper, despite the mean conductive area (34%) did not significantly differ between the two species (Table 1). The chronologies based on these anatomical variables showed lower correlation values between conspecific trees as compared with tree-ring width series, except for Kh. The annual mean production of IADFs in Spanish juniper was twice (32%) that in Aleppo pine (15%), but the mean IADF series of both species were highly related ($r = 0.33$, $P = 0.04$) indicating that they encoded similar climatic information.

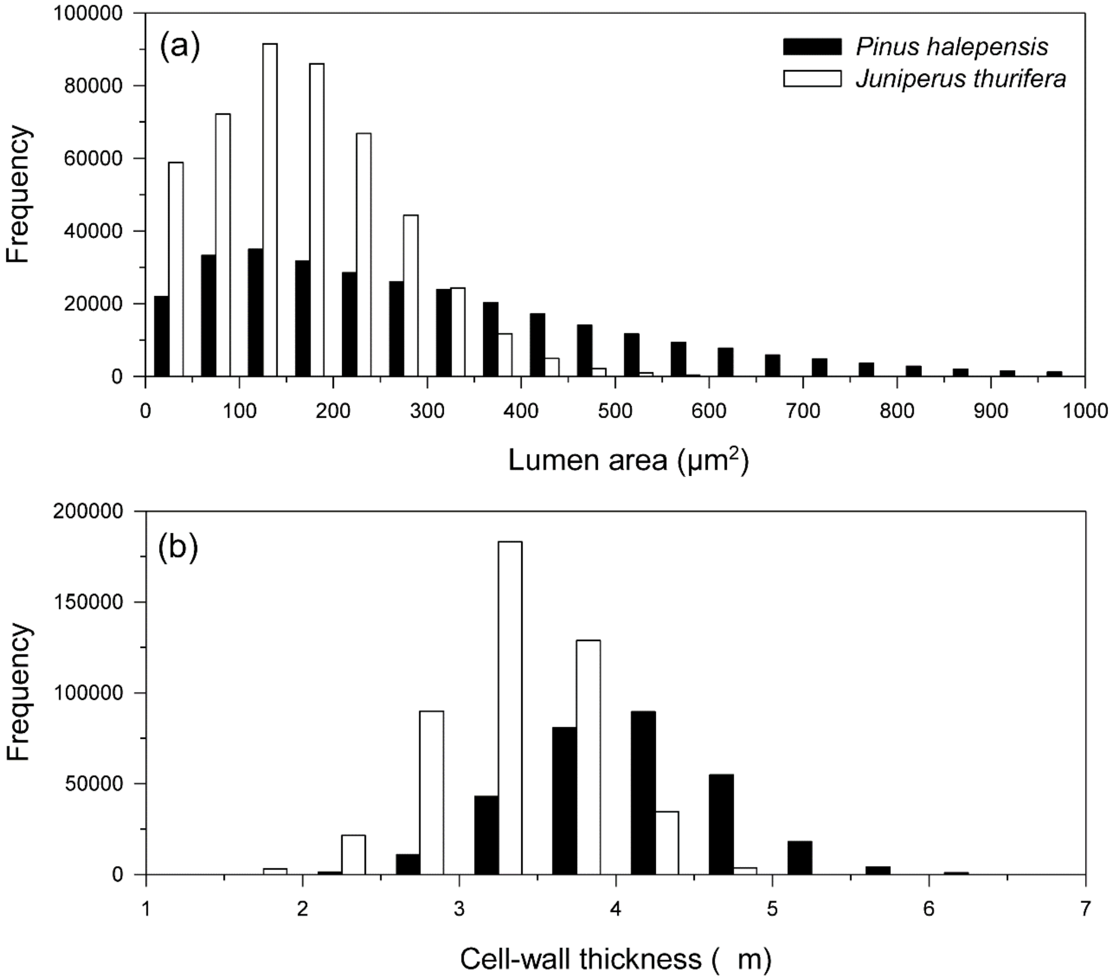

Figure 2.1 Distribution of (a) tracheid LA and (b) CWT for the two conifers (Aleppo pine, *P. halepensis*; Spanish juniper, *J. thurifera*). Data correspond to the 1975-2013 period.

We found a highly significant (P<0.001) relationship between tree-ring width and the number of tracheids forming a ring, slightly stronger in juniper (r = 0.93, n = 244) than in pine (r = 0.90, n = 179). As expected, for a similar number of tracheids pine formed rings approximately twice the width of those of juniper (Online Resource 4, Fig. S4). In addition, dh and CWT were negatively related in juniper (r = -0.40, n = 237, P = 0.015), whereas this association was positive in pine (r = 0.26, n = 176, P < 0.001).

Table 2.1 Main descriptive variables obtained for the trees considering size, age, tree-ring width and wood-anatomical variables. Values are means and standard deviation calculated for the period 1970-2013. Different letters indicate significant ($P<0.05$) differences between species based on Mann-Whitney U tests.

Variables	Spanish juniper (*Juniperus thurifera*)		Aleppo pine (*Pinus halepensis*)	
	Variable	Correlation between trees	Variable	Correlation between trees
Diameter at 1.3 cm (cm)	14.8 ± 1.0	−	16.7 ± 1.0	−
Tree height (m)	5.1 ± 0.2a	−	6.0 ± 0.2b	−
Age at 1.3 m (years)	60 ± 15	−	54 ± 12	−
Tree-ring width (mm)	1.26 ± 0.72	0.42	1.82 ± 1.14	0.53
Frequency of IADFs (% year^{-1})	32 ± 20b		15 ± 12a	
No. tracheids per ring	4264 ± 1053		2966 ± 835	
LA (μm^2)	147.26 ± 36.55a	0.14	275.78 ± 86.46b	0.22
CWT (μm)	3.43 ± 0.31a	0.28	4.14 ± 0.36b	0.10
d_h (μm)	17.00 ± 1.97a	0.30	24.82 ± 4.48b	0.39
K_h (kg m MPa^{-1} s^{-1} 10^{-13})	6.22 ± 3.93a	0.45	12.50 ± 7.59b	0.60
Conductive area (%)	33.58 ± 6.34	0.24	34.44 ± 5.47	0.25

Abbreviations: IADF, intra-annual density fluctuation; LA, lumen area; CWT, cell-wall thickness; dh, hydraulic diameter; Kh theoretical hydraulic conductivity.

2.4.2 Phenology of radial growth and xylem formation

On average, both species started growing in early April and finished in late October (Table 2). Maximum radial-growth increment rates were observed in mid (Spanish juniper) to late May (Aleppo pine), whilst maximum stem decrement rates were detected in early (Spanish juniper) to late August (Aleppo pine) in response to summer drought. Xylogenesis data showed that the first tracheids were formed in late March in both species, i.e. almost 15 days before the first stem radial increment was detected (Table 2). However, the maximum xylem growth rates, corresponding to the formation of radially-enlarging tracheids, occurred in mid- to late May, and they almost coincided with those observed using dendrometers. The wall-thickening tracheids started and finished forming from early May to mid-October in Aleppo pine, and from late May to late October in Spanish Juniper.

Table 2.2 Dates (day of the year) summarizing the xylem phenology (xylogenesis) of the two studied species (Spanish juniper, *Juniperus thurifera*; Aleppo pine, *Pinus halepensis*) in the study site based on manual band dendrometers (2006-2012 period) or xylogenesis (2006 and 2010) data.

Year	Dendrometer data								Xylogenesis data							
	Juniperus thurifera				*Pinus halepensis*				*Juniperus thurifera*				*Pinus halepensis*			
	F	MG	MD	L	F	MG	MD	L	EE	ME	EL	LL	EE	ME	EL	LL
2006	112	163	201	334	110	175	195	304	95	133	142	315	75	155	135	290
2007	115	141	200	292	90	134	257	290								
2008	102	136	247	302	103	152	255	307								
2009	90	135	205	272	89	135	225	330								
2010	97	131	183	321	114	160	257	322	88	137	150	290	88	142	122	285
2011	94	124	257	331	90	134	260	290								
2012	103	132	222	278	94	131	221	296								
Mean	102	137	216	304	99	146	239	306	92	135	146	303	82	149	129	288
SD	30	37	34	17	10	17	25	16	5	3	6	18	9	9	9	4
Julian day	11 Apr	16 May	3 Aug.	30 Oct.	7 Apr.	24 May	25 Aug.	31 Oct.	31 March	14 May	25 May	28 Oct.	21 Mar.	27 May	7 May	13 Oct.

Abbreviations referring to dendrometer data: F, first positive growth rate, MG, maximum radial increment rate; MD, maximum radial decrement rate; L, last positive rate.

Abbreviations of xylogenesis phases: EE, start of growth (first enlarging tracheids are formed); ME, maximum production of radially-enlarging tracheids; EL, beginning of wall-thickening tracheids; LL, last wall-thickening tracheids are formed.

2.4.3 Inter-annual temporal patterns in growth and wood anatomy

Raw chronologies of tree-ring width and LA showed negative and positive significant ($P<0.001$) trends, respectively, in both species (pine, $r = -0.45$ and $r = 0.77$; juniper, $r = -0.62$ and $r = 0.46$). The year-to-year variability in ring width was similar between the two species ($r = 0.73$, $P<0.001$) indicating a likely common climatic signal (Fig. 2). In fact, growth peaked in years characterized by wet spring conditions (e.g., 1997) whilst wood production was very low during years with a warm and dry spring (e.g., 2005; see Online Resource 1). The patterns of mean LA, per ring, were also consistent over time between the two coexisting species ($r = 0.71$, $P<0.001$) but this was not the case for CWT ($r = 0.23$, $P<0.001$).

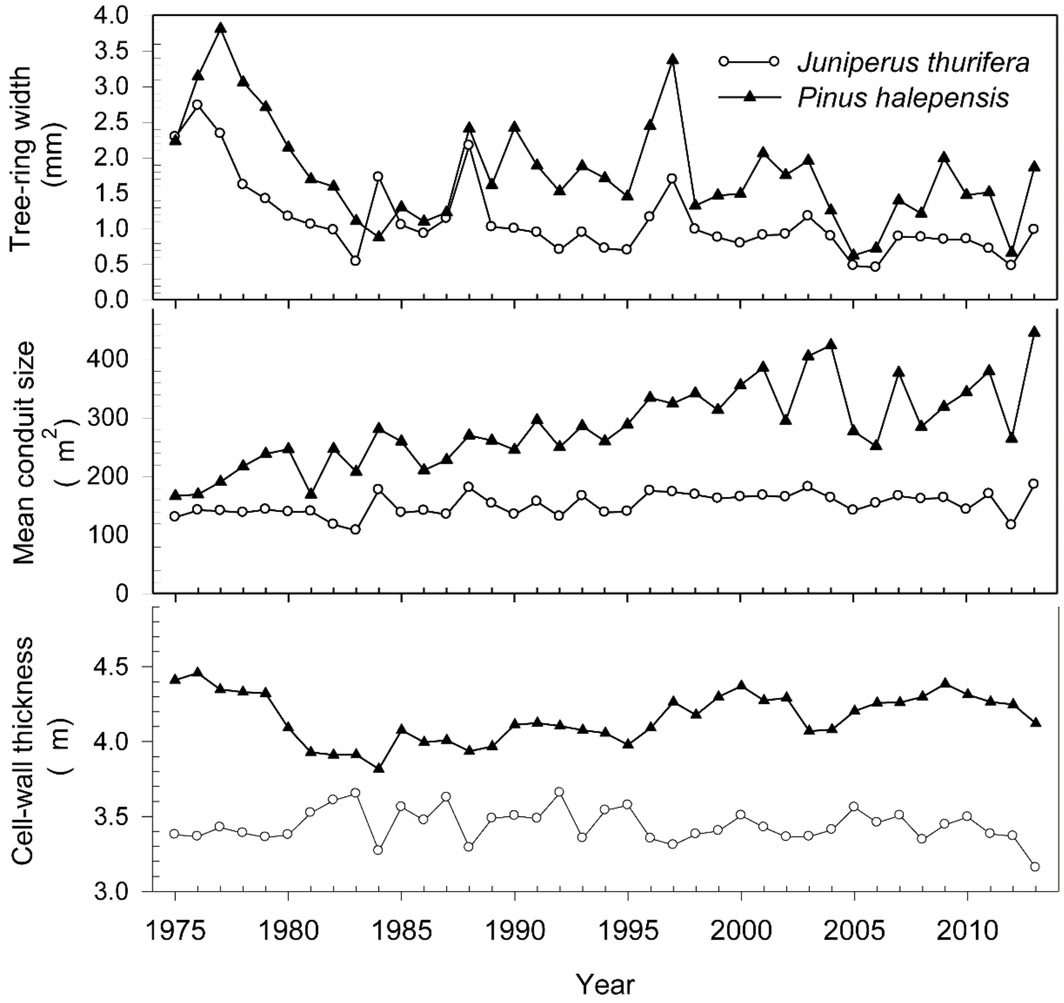

Figure 2.2 Mean tree-ring width, lumen area (mean conduit size) and cell-wall thickness chronologies for the two species (Spanish juniper, *Juniperus thurifera*; Aleppo pine, *Pinus halepensis*).

2.4.4 Climate influences on wood anatomy

The results obtained using 15-day window produced the most robust and significant correlations. In general wet and cool conditions during the previous winter (recharge of soil water) and in spring (Spanish juniper) or summer (Aleppo pine) enhanced lumen area expansion, whilst dry spring-to-summer conditions (negative water balance) reduced conduit size (Online Resource 5, Fig. S5). Warm summer to autumn conditions preceded by low SMD enhanced CWT in the case of Spanish juniper, whereas wet and warm late-winter and summer conditions were associated to high CWT values in Aleppo pine. The first of these two associations was also observed in juniper. Considering the analyses based on tree-ring deciles, wet conditions from February to July enhanced LA of tracheids located in the first four deciles (early to mid earlywood) and also in the last decile (late latewood) in both species, with maximum positive associations observed in May and June (Fig. 3). Contrastingly, the positive role of warm February conditions on LA affected most deciles distributed throughout the ring in juniper, but mainly the 5th to 6th deciles in the pine (mid to late earlywood). In the case of CWT, warm February minimum temperatures enhanced wall thickening in Aleppo pine along the entire ring but the signal was stronger in the first three and last two deciles. Wet February to March conditions also enhanced CWT in both species, but July to September wet conditions only increased CWT in the last two deciles (latewood) of pine tracheids.

2.4.5 Climatic drivers of IADF formation

A positive water balance in late July (pine) or early September (juniper) increased the formation of IADFs in the latewood (Fig. 4). However, cold conditions in March to June and in autumn (only in juniper) were negatively related to IADF formation. Lastly, a higher SMD in autumn was associated to the production of IADFs, being more important from September to October in the case of juniper. Considering monthly values, in pine IADF frequency was positively related to wet July to August conditions, whilst in juniper IADFs were formed in response to years with wet (high water balance and low VPD) August conditions (Fig. 5).

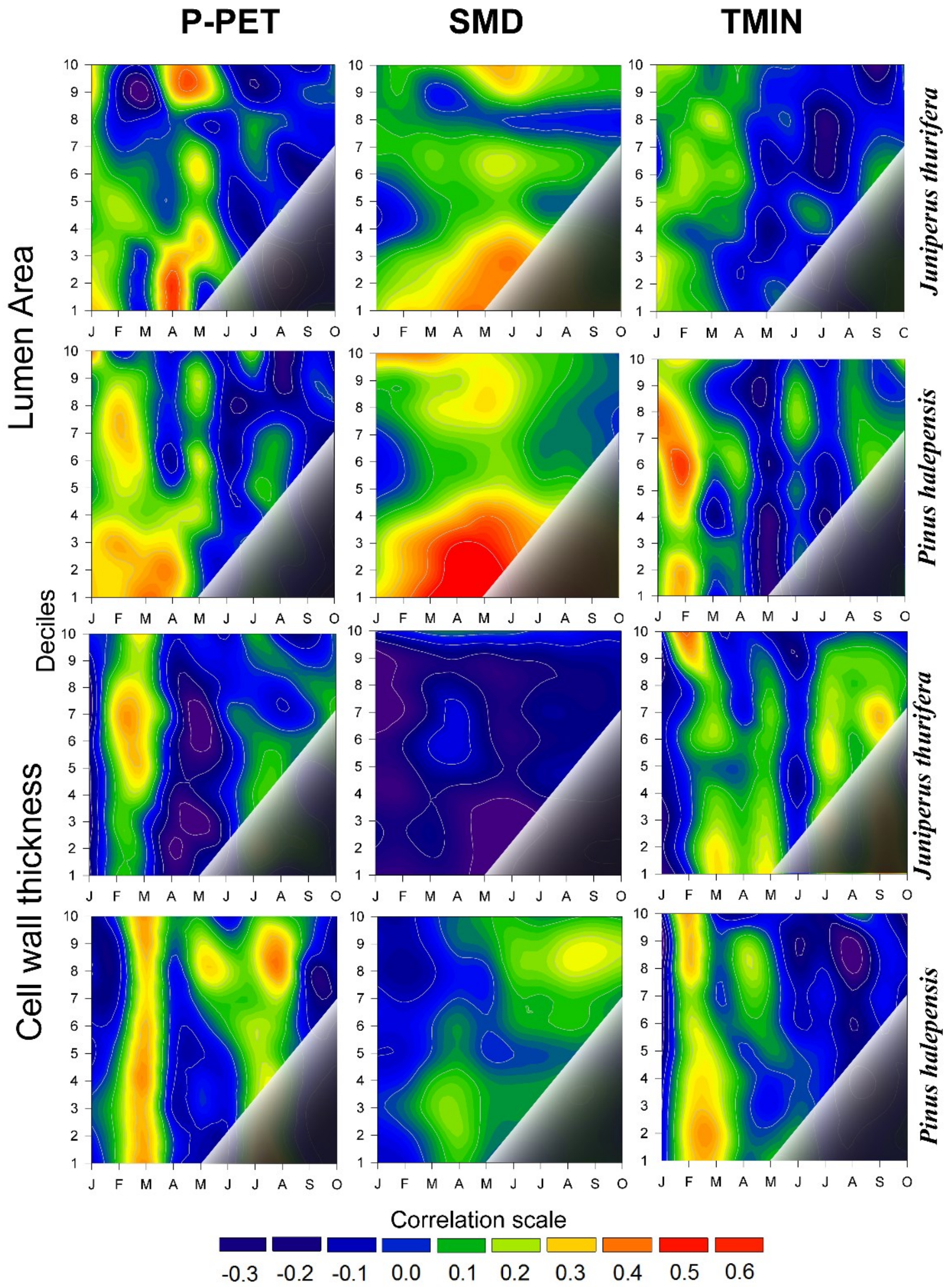

Figure 2.3 Climate-growth associations computed by comparing lumen area and cell-wall thickness with monthly climatic variables (P-PET, water balance; SMD, soil moisture deficit; and Tmin, minimum temperature) in Spanish Juniper (*Juniperus thurifera*) and Aleppo pine (*Pinus halepensis*). Correlations were calculated from January to October of the year of tree-ring formation (x axes) and partitioning the tree-ring in deciles along the radial direction from the beginning (decile 1) to the end (decile 10) of the ring (left y axes). Correlations coefficient above │0.31│ are significant at P<0.05. Chronologically inconsistent correlations (bottom-right triangle in each plot) are not shown.

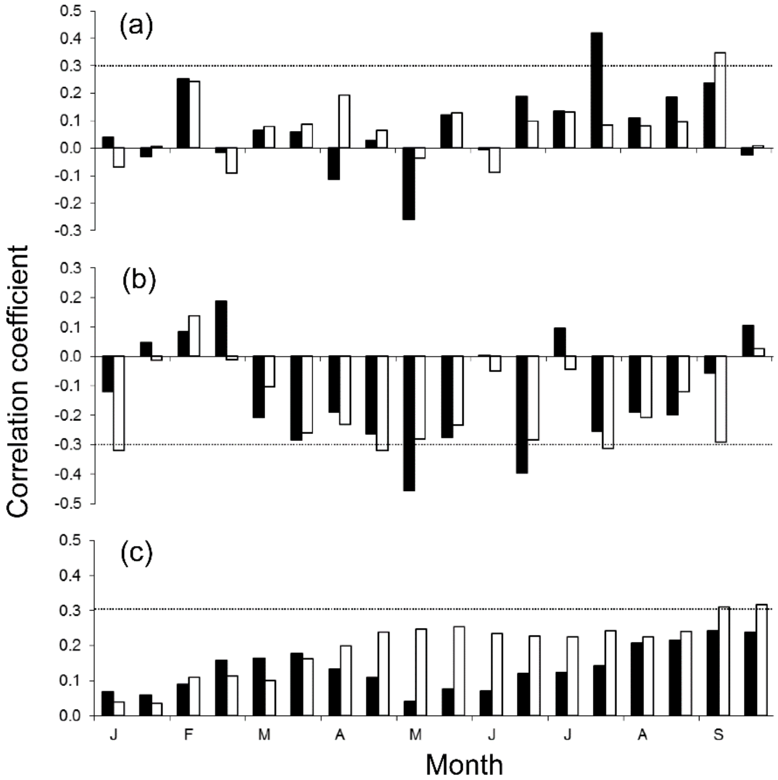

Figure 2.4 Spearman correlations computed for the two species (filled bars, Aleppo pine, *Pinus halepensis*; empty bars, Spanish juniper, *Juniperus thurifera*) by relating the frequency of intra-annual density fluctuations and the 15-day summed water balance (a), averaged mean minimum temperatures (b) and summed soil moisture deficit (c). Horizontal lines indicate significance levels at $P < 0.05$.

2.5 Discussion

Here we evidence that wood-anatomical features encode long-term and high-resolution climatic information on tree functioning and growth of two conifer species coexisting under continental Mediterranean conditions. The observed relationships between climate and anatomical traits confirm previous information on xylogenesis of the two species. This allowed the major wood responses to water shortage to be pinpointed. We found that wet conditions during the early growing season in spring or before (winter) result in the formation of wide tree rings that consist of numerous tracheids with relatively large lumens in the case of pine, while lumen areas are smaller in juniper. The small lumen areas of juniper species make them less vulnerable to cavitation as compared with coexisting pine species forming tracheids with larger lumens (Willson et al. 2008). This seems to explain why drought-induced die-off mainly affects pines in mixed pine-juniper forests on semi-arid sites (McDowell et al. 2008). Although not just tracheid dimensions but also small alterations on pit traits (frequency, geometry and biomechanics) between coexisting species can affect tree performance on water transport, embolism resistance and capacitance (Hacke 2015). Considering only our measurements, wood-anatomical data therefore shows that junipers will be less vulnerable to die-off than pines since many Cupressaceae have evolved towards a drought-resistant xylem (Pittermann et al. 2012). On the contrary, pines will show higher growth rates and Kh if conditions are wet enough in the early growing season.

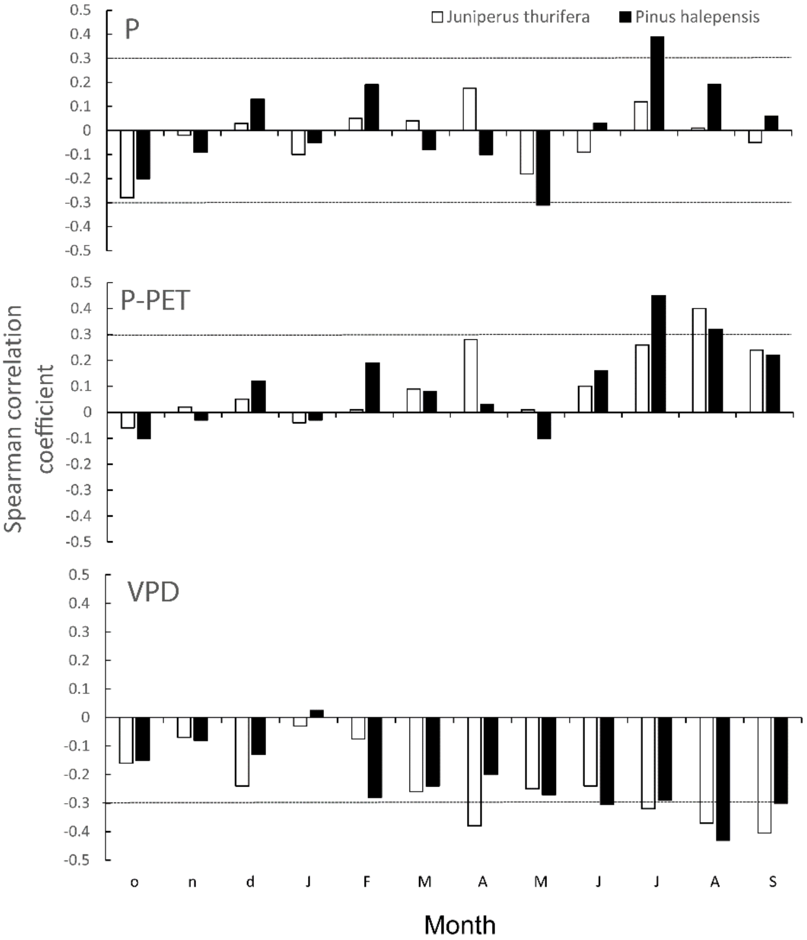

Figure 2.5 Relationships calculated between the frequency of latewood intra-annual density fluctuations (IADF) of Aleppo pine (*Pinus halepensis*, filled bars) and Spanish juniper (*Juniperus thurifera*, empty bars) vs. monthly climatic variables: precipitation (P), water balance (P-PET) and vapour pressure deficit (VPD). Climatic variables include the time window from previous October to current September. Lowercase and uppercase letters abbreviate months prior or concurrent to the growing season, respectively. Bars located outside the horizontal lines indicate significant correlations (P < 0.05).

As it ages Aleppo pine forms wider tracheids compared to Spanish juniper. This is also related to the usual bigger size on adult trees (tree height and deeper root system (Willson et al. 2008)).

The shallower root system of junipers could make them more dependent on summer and autumn rains than Aleppo pine, and this could explain why junipers tend to form latewood IADFs more frequently (Table 1) whereas pines are able to sustain low growth rates during the summer dry period (see also Camarero et al. 2010). This agrees with the different microsite location of the species on the study site. The soil water conditions play a central role from late March to June for the expansion of tracheid lumens in both species (Fig. 3). However, the responsiveness of lumen area to soil moisture deficit lasted until summer in juniper. In the case of cell-wall-thickness, correlations with climate were more variable than in the case of lumen area and differed between the two species. In both species, wet and warm late-winter conditions were associated to thicker earlywood and latewood tracheids confirming lagged climate-anatomy relationships since latewood starts forming from the end of May onwards (Table 2). Warm summer to autumn conditions preceded by low soil moisture enhanced cell-wall thickness in the case of Spanish juniper, whereas wet summer conditions were associated to thick cell walls in Aleppo pine. Such a response has been explained by the low intercellular variability in wall thickness among neighbouring tracheids in Spanish juniper (Olano et al. 2012). These authors attributed the formation of latewood tracheids with smaller lumen areas in this species to limited carbohydrate availability because of competition with other carbon sinks (Oribe et al. 2003). However, our interpretation is that the amount of carbon invested for each tracheid is fairly similar all over the growing season and the change in lumen area from earlywood to latewood is mainly responsible for the changes in cell-wall thickness which could represent an allometric relationship related to constraints of conduit size and wall reinforcement (Hacke et al. 2001), and not a carbon-related limitation of xylogenesis (Cuny et al. 2014). This argument is also supported for the pine species based on the associations observed between lumen area or hydraulic diameter and cell-wall thickness (Online Resource 4, Fig. S4).

Soil water availability during the early growing season, which depends on precipitation but also on soil temperatures and water-holding capacity, is one of the main drivers of wood formation and radial growth under continental Mediterranean climates (Martin-Benito et al. 2013). We found that wet and cool May conditions at a 15-day scale enhanced lumen expansion in both species as a result of improved cumulative soil water reserves, but high precipitation in June was associated to narrow lumens, particularly in Aleppo pine (Online Resource 5, Fig. S5). At a monthly scale, Aleppo pine formed larger lumens in response to a high water balance during the early spring when xylem formation starts (Fig. 3, Table 1), which is a similar signal to that observed for the tree-ring width (Pasho et al. 2012). This implies that those climatic conditions favourable to lumen enlargement also enhance radial growth and suggests that wood formation and a higher Kh in the early growing season are interrelated and largely determine the annual growth rate in this species. These findings confirm the importance of winter soil-water recharge for Aleppo pine prior to cambial resumption in spring and also suggests that this species can reach deeper soil-water pools than those accessible to junipers (Sarris et al. 2013). This vertical segregation of soil water resources would explain the observed growth behavior differences by allowing a minimal growth of Aleppo pine in summer, whilst making juniper more dependent on spring rainfall to grow (Fig. 3).

The longer season of latewood formation would explain a most active cambial activity of juniper in autumn and the frequent formation of IADFs. The positive effect of spring precipitation and summer SMD on the lumen area of juniper latewood tracheids indicates that the main driver of IADFs in this species is an increase in the water balance and a decrease on vapour pressure deficit, i.e. a reduction in soil and atmospheric dry conditions, which leads to the formation of tracheids with ample lumen areas in late summer and early autumn. This clearly represents a carry-over effect in juniper response having first the punctual events of spring precipitations inducing a long lasting effect on soil moisture, which finally is permitting the species to produce the second peak of cambial activity. Contrastingly, latewood IADF formation in Aleppo pine is mainly favored by high precipitation in mid-summer in agreement with its earlier latewood development (Camarero et al. 2010).

The formation of latewood IADFs represents a valuable ecological proxy of late-summer to early-autumn water availability, although this is still little explored and IADF formation is not fully mechanistically understood. The relatively rapid formation of wide-lumen tracheids within the latewood suggests that the cambium, apparently dormant during the summer drought, reacts quickly to a significant amount of precipitation by forming an IADF. However, this response is similar to what is observed in the earlywood despite both occurring in completely different xylogenetic phases, namely radial enlargement of earlywood tracheids in the early growing season vs. active lignification and maturation in the late growing season.

To conclude, tree-ring width and lumen area responded to wet previous winter to current spring conditions, respectively, in two conifers of different genera coexisting under continental Mediterranean conditions. The formation of latewood IADFs was favored by late-summer to early-autumn wet conditions which triggered the formation of tracheids with ample lumen areas. The relationships found between climate, growth and wood-anatomical data concur with the bimodal pattern of xylogenesis described for conifers in continental Mediterranean climates. The plastic responses of wood-anatomical variables to climate, and particularly to water deficit, could be caused by the different accessibility of soil water reserves for the species since junipers develop more shallow root systems than pines.

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2.7 Supplementary Material

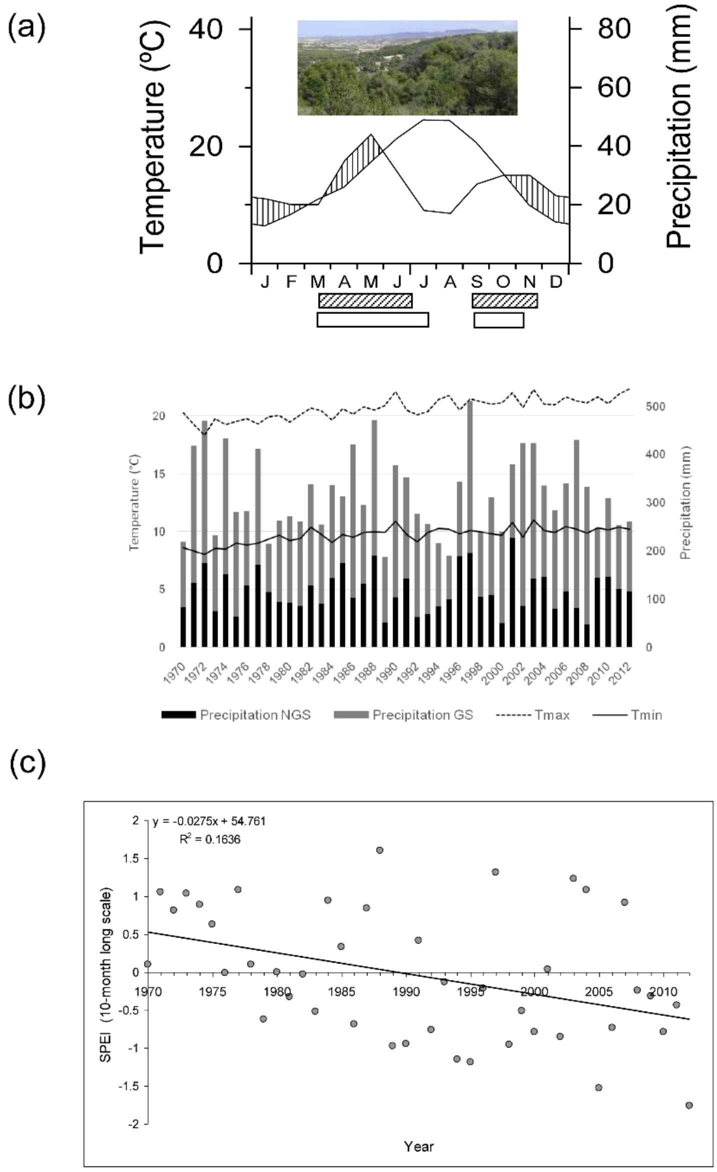

Figure S 2.1 Climatic diagram and view of the study site (a) and climate trends for the period 1970-2012 (b, c). In the uppermost plot dashed and empty lower bars indicate the periods of active xylogenesis for Spanish juniper (*Juniperus thurifera*) and Aleppo pine (*Pinus halepensis*), respectively (Camarero et al. 2010). In the (b) plot, total precipitation is divided in growing (GS, April to October) and non-growing Season (NGS, November to March), whilst temperatures are presented as mean monthly maximum and minimum values. The lowermost plot shows the trends of the Standardised Precipitation-Evapotranspiration Index (SPEI), calculated for the GS and considering 10-month long scales (cf. Pasho et al. 2012). This drought index shows a trend towards drier conditions in the study site (more negative SPEI values).

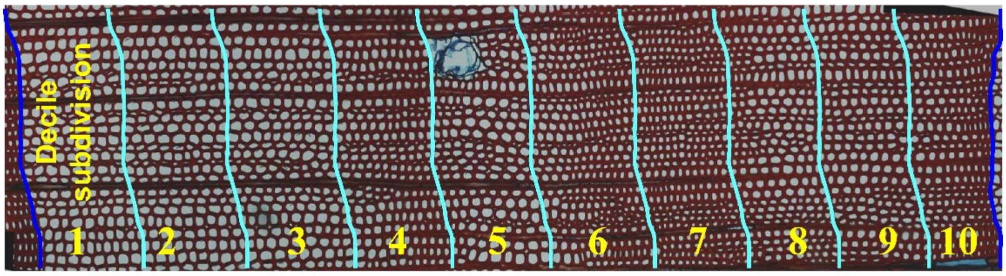

Figure S 2.2 Graphical representation of tree-ring partitioning along the radial direction using 10 equal sectors or deciles.

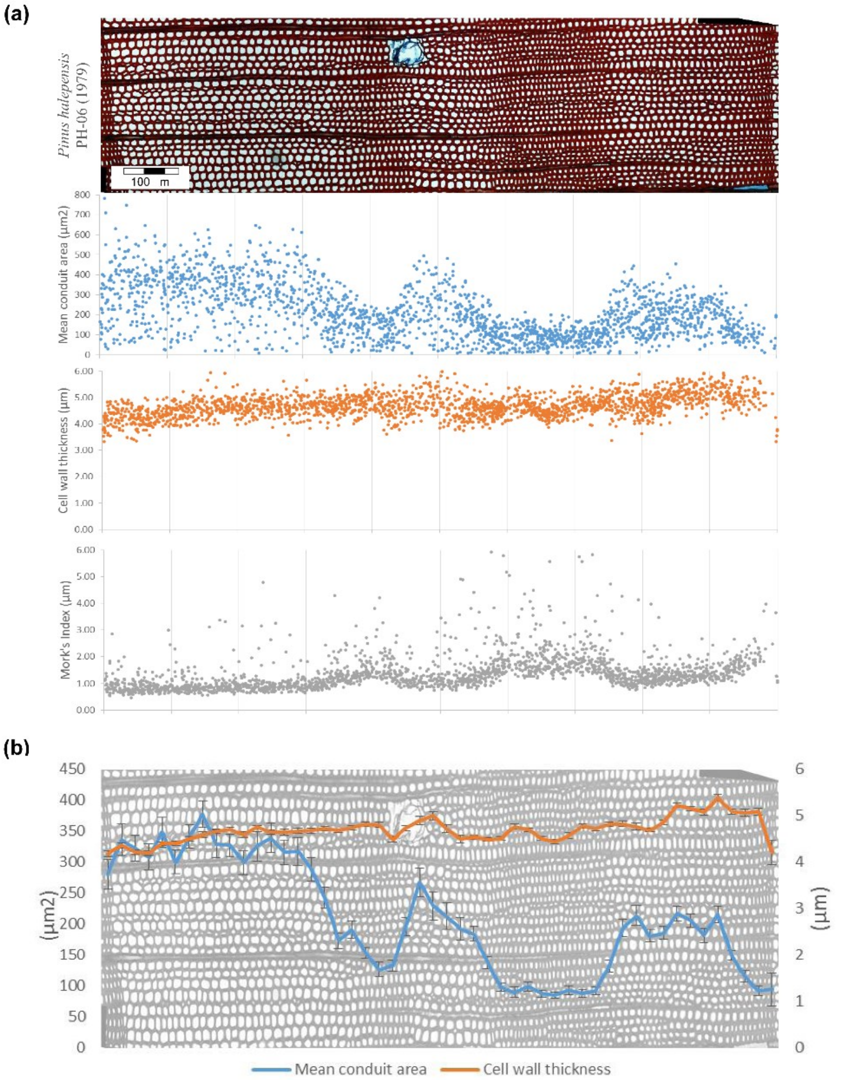

Figure S 2.3 (a) Compared values of mean conduit or lumen area, cell wall thickness and Mork's index as scatter plots against their corresponding source of data image. (b) Mean conduit area and cell wall thickness mean values appear overlapped on the photograph of a tree ring. In both cases data correspond to ring formed in 1979 by the Aleppo pine (*P. halepensis*) number 6.

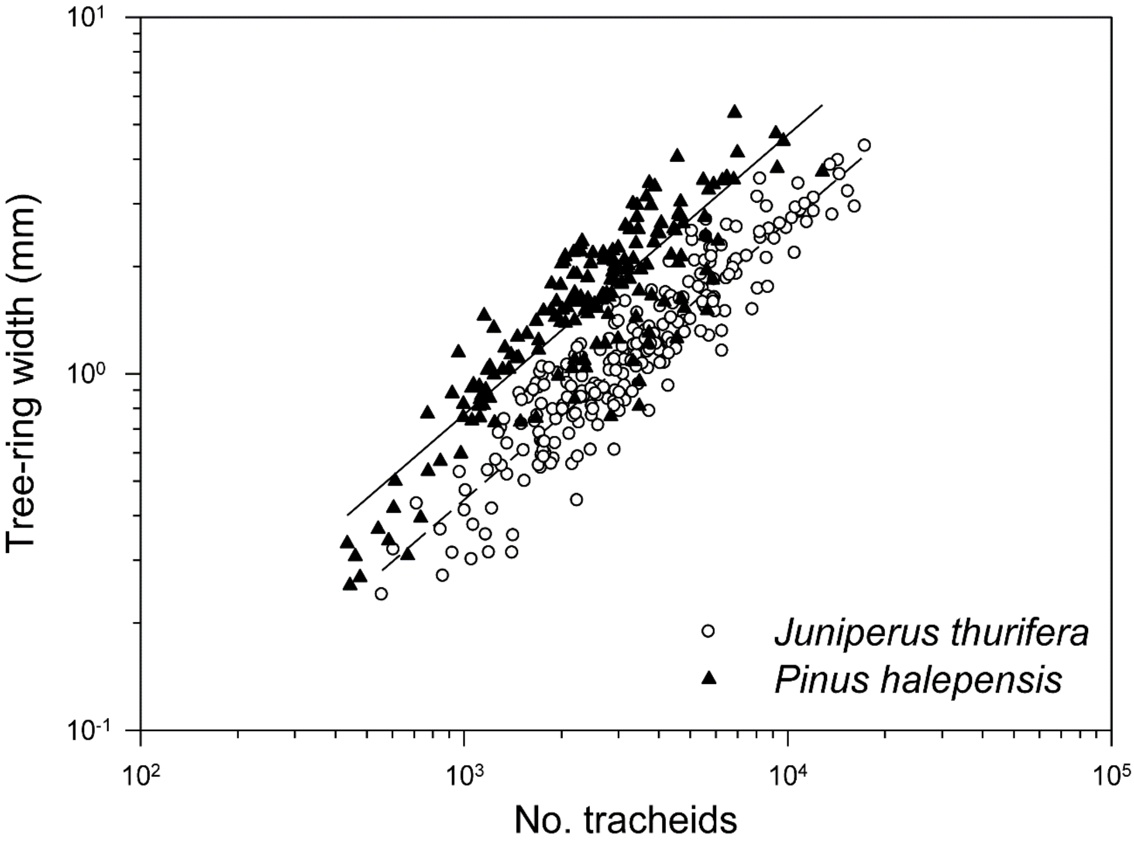

Figure S 2.4 Main relationships observed between growth and anatomical features of the two species (triangles: Aleppo pine, *Pinus halepensis*; circles: Spanish juniper, *Juniperus thurifera*).

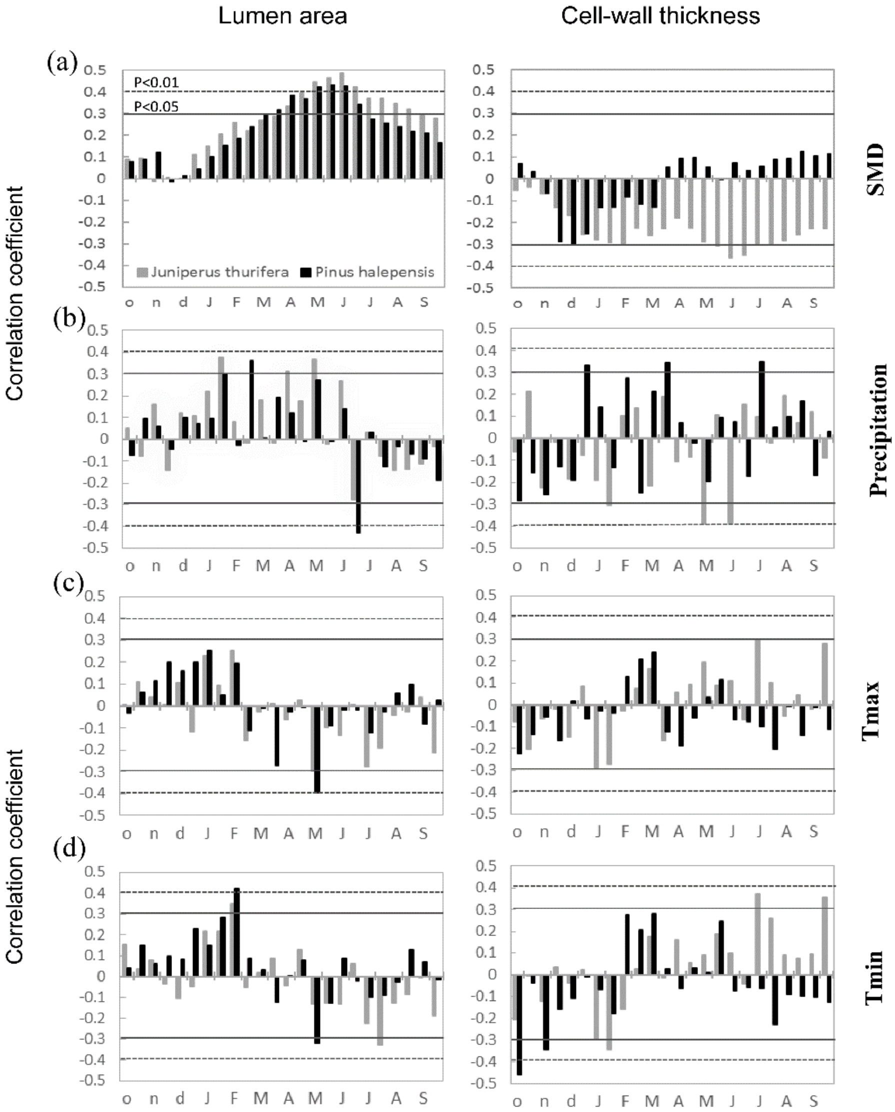

Figure S 2.5 Correlations calculated between lumen area (left columns) and cell-wall thickness (right columns) of Aleppo pine (*Pinus halepensis*, black bars) and Spanish juniper (*Juniperus thurifera*, grey bars) vs. several climatic variables: (a) soil moisture deficit (SMD), (b) precipitation; (c) mean maximum (Tmax) and (d) minimum (Tmin) temperatures. Climatic variables were either summed (a, b) or averaged (c, d) for 15-day periods and considering the time windows from previous October to current September. Correlations were run considering the whole ring length. Months prior or concurrent to the growing season are abbreviated by lowercase and uppercase letters, respectively. Continuous and dashed lines indicate *P*< 0.05 and *P*< 0.01 significance levels, respectively.

3. Disentangling the climate-driven bimodal growth pattern in coastal and continental Mediterranean pine stands

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KEYWORDS: intra-annual density fluctuation, *Pinus halepensis*, quantitative wood anatomy, tracheid lumen, xylem, wall thickness.

3.1 Abstract

Mediterranean climate promotes two distinct growth peaks separated by summer quiescence in trees. This bimodal pattern has been associated to favourable growing conditions during spring and autumn when mild temperatures and soil-water availability enhance cambial activity. Climatic models predict progressive warming and drying for the Mediterranean Basin, which could shorten or shift the spring and autumn growing seasons. We explored this idea by comparing two sites with different Mediterranean climate types (continental/dry and coastal/wet) and studied how climate drives the bimodal growth pattern in Aleppo pine (*Pinus halepensis*). Specifically we investigated the intra-annual changes in wood anatomy and the corresponding formation of density fluctuations (IADF). Trees on both sites were analyzed by dendrometer monitoring and by developing chronologies of wood anatomical traits. Radial-increment dynamics followed a similar bimodal pattern in both sites but coastal trees showed higher increments during the spring and autumn growth peaks, especially in autumn. The summer rest of cambium activity occurs almost one month earlier in the coastal than in the inland site. Lumen area and cell-wall thickness were significantly smaller in the continental site, while the increment rate of cell-wall thickness during an IADF event was much higher in the coastal pines. The accumulated soil moisture deficit was the main climatic constraint of tracheid enlargement in continental pines. Intra-annual density fluctuations were more frequent in the coastal trees where wood anatomy features recover to average values after such events, meanwhile inland trees presented a much lower recovery rate. Growth bimodality and the formation of density fluctuations were linked, but mild climate of the coastal site allows a longer growing season, which explains why trees in this area showed higher and more variable growth rates.

3.2 Introduction

In Mediterranean regions of the northern Hemisphere many tree species present, within the same growing season, two periods of growth activity, one in spring and a second in autumn. This bimodal pattern has been associated to favourable growing conditions, like mild temperatures and adequate soil-water availability (Mitrakos 1980). In between these growth periods, low winter temperature and summer drought can limit or even pause cambium activity (Ribas 2006, Camarero et al. 2010, Gutiérrez et al. 2011, Touchan et al. 2012, Prislan et al. 2016). This growth rest allows trees to acclimatize to summer droughts, which, although very variable in duration and intensity between regions and years, are typical of Mediterranean climates and greatly affect forest productivity (Girard et al. 2012).

This bimodal growth pattern can usually leave a permanent imprint in xylem with the formation of a distinctive anatomical wood features such as intra-annual density fluctuations (hereafter IADFs). These tree-ring features (Fritts 2001, De Micco et al. 2014) represent a relevant wood trait to identify bimodality in Mediterranean tree species (Cherubini et al. 2003). IADFs are formed when limiting conditions interrupt normal radial growth, producing latewood-like cells (usually with narrow lumens and thick walls) within the earlywood or, more frequently, earlywood-like cells (usually with wide lumens and thin walls) within the latewood (Campelo et al. 2007, Zalloni et al. 2016). As for most xylem anatomical traits, IADFs are typically the result of trade-offs between competing demands for growth, stem support, carbon storage, and transport of water and nutrients under changing climatic conditions (Vaganov et al. 2006, Zweifel et al. 2006). Differences in tree size and age can also play a role to determine the resulting IADF features (Campelo et al. 2013, 2015). IADF formation is not necessary linked to bimodal growth pattern; for example, Rigling et al. (2003) found that IADF formation was driven by cool and moist summer conditions in dry Alpine sites. In eastern Spain, dry conditions induce a reduction of summer growth rate in Aleppo pine followed by a reactivation of cambial activity during rainy autumns which often leads to the formation of IADFs (De Luis et al., 2011, 2007, Camarero et al. 2010, Pacheco et al. 2015). Nevertheless, under highly variable climatic conditions and with the hysteresis of some growth processes, it is possible to have significant site- and species-specific effects (Novak, et al. 2013, Novak, et al. 2013, Battipaglia et al. 2016) together with significant delays between the triggering climatic factor and its translation into the wood anatomical imprints (Camarero et al. 1998).

Stem radial growth and xylem phenology and development (xylogenesis) are usually assessed with band or point dendrometers and repeated wood-sampling. Both these intensive monitoring approaches proved to be very effective to thoroughly investigate causal relationships between environmental variability and cambial dynamics across many different biomes and tree species (Rossi et al. 2016). However, most of these researches last for relatively short periods, often encompassing less than five years. For this, in areas with a significant year-to-year climate variability, the risk is to monitor beside growth, the seasonal stem-water content fluctuations combined with idiosyncratic site and climate conditions of the sampling years (Drew and Downes 2009). This limitation in the temporal length of the investigations is very common in the drought-prone Mediterranean areas, where there are still very few studies on radial-increment dynamics longer than five years providing a thorough interpretation on climate-growth relationships (but see Ribas 2006, Gutiérrez et al. 2011). This calls for a better understanding of the weather settings that cause IADF formation if we aim to use them as bimodality markers in Mediterranean tree species. To this end we proposed a different approach straggling between the typical short-term intensive monitoring adopted tracking cambial activity with xylogenesis or dendrometers monitoring and the long-term retrospective investigation applied with tree-ring analysis. Here we still consider dendrometer records but we adjoined this short-term information with dendroanatomical analysis to untangle the high-resolution but long-term information encoded in tree rings (Gartner et al. 2002, Fonti et al. 2010, von Arx and Carrer 2014). A previous pilot investigation, applying this innovative dendroanatomical approach, has already successfully assessed intra-annual growth fluctuations in a long-term context. This helped to match short-term cambial activity monitoring with long-term (six decades) wood-anatomical time series and to improve the interpretation of the climate/growth relationships (Pacheco et al. 2016).

Aleppo pine (*Pinus halepensis* Mill.) is the most widespread tree species in the Mediterranean Basin lowlands (Ne'eman and Trabaud 2000). It tolerates many soil types and grows under very diverse climatic conditions, from continental inland sites to mild conditions close to the coastal areas, but generally subjected to summer drought (Barbéro et al. 1998). It is also a fire-adapted, shade-intolerant species that is able to withstand dry conditions (Borghetti et al. 1998). These characteristics make Aleppo pine the most suitable conifer tree species for studying the effects of summer drought within the context of climate change in the Mediterranean. We selected two Aleppo pine stands located in eastern Spain and subjected to contrasting Mediterranean climate types: a continental site located near Zaragoza and a mild coastal site near Barcelona. We expect that changes on stem diameter and wood-anatomical features (i.e. lumen area and cell wall thickness) will describe similar patterns showing the bimodal growth, but also to find differences in the response to seasonal changes in water availability between drier and wetter

Mediterranean sites. We also hypothesize that any difference found in intensity of the bimodal growth patterns and frequency of IADFs between sites will be reflected at the wood anatomy level.

Our specific aims were (1) to describe and compare the intra-annual patterns of stem radial growth captured by each methodology in Aleppo pine at both study sites, (2) to assess whether the climatic signal in anatomical traits mirrors the bimodal growth pattern shown by the dendrometers, and (3) to determine which previous and intra-seasonal climatic conditions are triggering the formation of IADFs.

3.3 Materials and methods

3.3.1 Study sites and climate

The study was conducted in two locations with mild (wet) and continental (dry) Mediterranean climatic regimes situated in coastal and inland sites, respectively. For the coastal Mediterranean site, we selected an interior valley near Barcelona (northeastern Spain) on the Garraf karstic mountains (41° 20' N, 1º 50' E) in the central Mediterranean coastal ranges at an altitude of 300 m a.s.l. in the Natural Park of Garraf and Olèrdola. Vegetation cover is around 95% and is mainly comprised of *P. halepensis* with *Quercus coccifera* L. maquis dominating southern slopes and *Q. ilex* L. on the northern ones (Gutiérrez et al. 2011). The soil type is fersiallitic, decarbonated and argillic, which makes it susceptible to compaction. The presence of rocky outcrops creates a shallow and discontinuous soil surface with a low water-holding capacity (Ribas 2006, Gutiérrez et al. 2011). According to climatic data from the nearest weather station at Begues (ca. 8 km from the study site), mean annual temperature for the period 1950–2014 was 13.2 ºC with a maximum mean monthly temperature of 21.1 ºC (July) and minimum of 5.1 ºC (January). Mean annual precipitation was 673 ± 174 mm, with 33% recorded during autumn (September to November), 25% during spring (March and May) and an estimated water deficit period spanning from June to August (Gutiérrez et al. 2011) (Supplementary material, Figure S1).

For the continental Mediterranean site we selected a mixed Mediterranean forest (Vedado de Peñaflor, 41º 47' N, 0º 43' W) near Zaragoza (Middle Ebro Basin, northeastern Spain) at an altitude of 340 m a.s.l. The terrain consists of small hills and valleys with west- and east-facing slopes, covered by a mixture of *P. halepensis*, *Juniperus phoenicea* L., *J. thurifera* L. and *Q. coccifera* L. among other woody species (Camarero et al. 2010). The soil at the site originates from a parent rock comprising gypsums and marls. Its depth is considered to be around 50 cm by observing trees uprooted by wind on the site (J.J. Camarero, personal observation). The climate, based on data from the nearest weather station at Aula Dei (ca. 5 km from site) during the period 1950 to 2014, is classified as continental Mediterranean with an average annual rainfall of 313 mm (semi-arid conditions). Annual mean temperature was 13.8 ºC with frosts occurring from December to January and maximum and minimum mean monthly temperatures of 25.0 ºC (July) and 6.4 ºC (January), respectively. The period with water deficit spans from March to October (Supplementary material, Figure S1).

3.3.2 Climate data

For both study sites there were weather stations nearby with fairly complete daily data available since 1952. For the Zaragoza site we used precipitation records from the Peñaflor-Aula Dei (41° 45’ N, 0° 49’ W, 280 m a.s.l.) and temperature records from Zaragoza-airport (41° 40’ N, 1° 00’ W, 263 m a.s.l.) weather station located at 30 km from the study site. At the Barcelona site we used all daily data from the Barcelona-airport (41° 17' N, 2º 05' E, 1 m a.s.l.) weather station, some 15 km to the east of the study site. We considered the daily-resolved records for mean, maximum and minimum temperatures, relative humidity, and total precipitation for the period 1952-2012. Vapour pressure deficit (VPD) was estimated using temperature and humidity data (Abtew and Melesse 2013). We also calculated the water balance as the difference between precipitation and potential evapotranspiration (PET) following Hargreaves et al. (1982). The soil moisture deficit (SMD) was estimated as the difference between cumulative evapotranspiration and cumulative rainfall (Cocozza et al. 2012). Daily data were also averaged (temperatures) or summed (precipitation, water balance) at various time intervals (5, 10, 15, 20, 30, 40, 50 and 60 days) to analyze climate-growth relationships at multiple temporal scales following Gutiérrez et al. (2011).

3.3.3 Dendrometer measurements

Radial increment in Aleppo pine trees was monitored using stainless-steel manual band dendrometers (Agricultural Electronics, Tucson, USA). Dendrometers were placed at 1.3 m around the stem after brushing the dead bark. The site in Barcelona was divided into two groups of trees of different age. One group comprised 10 trees with an average age of 33 years and a measurement period extending from 1995 to 2004 (120 months), while a second group of 5 older trees (age = 75) was measured from 2001 to 2004 (48 months). In Zaragoza the period of data collection went from 2006 to 2014 (115 months) and was performed on one group of 10 trees with ages ranging from 35 to 75 years. For both sites dendrometer readings were taken up to twice per month during the growing season and main tree characteristics were measured at the beginning of the trial in Zaragoza (2006) and at the end in Barcelona (2004) (Supplementary material, Table S1). The cumulative circumference data, provided by the dendrometers, was converted to cumulative radial increment data assuming that the stem was cylindrical and dividing the girth data by 2π. For each tree, we calculated daily radial-increment rates by dividing the radial increment by the number of days between two consecutive observation dates.

3.3.4 Sample collection and wood-anatomy analyses

During the winters of 2014 and 2015, a total of 20 dominant trees (10 per site), from the same sites as the dendrometer trial, were randomly selected and sampled. At the Barcelona site the cores were taken from the older group including 4 trees with dendrometers, while in Zaragoza cored trees did not include those with dendrometers installed. We took three radial cores per tree at 1-1.3 m with Pressler increment borers 5 and 10-mm diameter. All cores were prepared following standard dendrochronological protocol (Stokes and Smiley 1968). The ring widths were measured to the nearest 0.01 mm using LINTAB measurement equipment fitted with a stereoscope and TsapWin software (Rinntech, Heidelberg, Germany). To ensure correspondence between each annual ring and the calendar year of ring formation, cross-dating was checked using COFECHA software (Holmes 1983). The 10-mm cores were then used for the histological analyses.

Cores selected for wood-anatomical analyses were divided into pieces 4-cm long, boiled in water to soften the wood and sliced into 10-12 μm thick transversal sections using a rotary microtome (Leica RM 2025, Heidelberg, Germany). The samples were stained with safranin (1%) and astrablue (0.5%) (both were diluted in distilled water) and rinsed with water and ethanol. They were then fixed on permanent slides with Eukitt (BiOptica, Milan, Italy) (von Arx et al. 2016). Histological images were obtained using a digital camera (Nikon Digital Sight DS-5M) mounted on a light microscope (Nikon Eclipse80i; Nikon, Tokyo, Japan); images were captured at 100X magnification. Overlapping images were taken from each sample and stitched together using the PTGui software (New House Internet Services, Rotterdam, NL) to obtain high-resolution images (0.833 pixels µm-1) of the entire section. Tree-ring borders were manually drawn on the images, which went through a semi-automatic analysis using the ROXAS software (von Arx and Carrer 2014). This analysis provided measurements (among others) on transversal lumen area (LA) and cell-wall thickness (CWT) (Prendin et al. 2017) of tracheids, and also gave the relative position of each tracheid within the dated annual ring.

3.3.5 Growth and wood-anatomy chronologies and relationships with climate

Chronologies of tree-ring width and wood-anatomical traits were produced for each site using ARSTAN. This software was developed to remove biologically induced age-size related growth trends (Cook 1985), which are also present in wood-anatomical data (Carrer et al. 2015). Individual series were fitted using a cubic smoothing spline function with 50% frequency–response cutoff of 30 years. Subsequently, observed values were divided by expected values to obtain detrended and dimensionless indices, which were then subjected to autoregressive modelling to remove the remaining serial autocorrelation. These residual or pre-whitened indices were averaged using the biweight robust mean to create the site chronology of each variable for the two species.

Using the relative position of each tracheid within each annual ring, we calculated mean values of LA and CWT along 100 equal-width tangential sectors to obtain a very detailed profile of anatomical features changes along the whole ring. To assess the associations with climate, we simplified the 100 sector scheme by averaging it into a 10 sector scheme (i.e. 10 equal-width tangential sectors per ring). These "deciles" helped us to better define the time windows over the phases of radial growth. The climate-growth associations were quantified by calculating Pearson correlations between wood-anatomical detrended chronologies and the different time windows of climatic data for the best-replicated period (1950-2014).

3.3.6 IADF analyses

IADFs were firstly identified visually and we considered only those characterized as earlywood-like cells within the latewood, which were more abundant at both study sites. After this identification, we calculated the presence (frequency %) of IADFs per year for each site (Supplementary material, Figure S3). The frequency of IADFs was assessed on a year basis as the percentage of trees showing a density fluctuation in any given ring. Then, to objectively compare sites we used the following protocol: i) we selected the years with the clearest single IADFs with at least a 50% frequency at site level, ii) using the minimum cell lumen area value of the density fluctuation as a center point, we split the tree ring profile into “pre-IADF” and “post-IADF” sectors, iii) we standardized the two portions of the profile using the tracheidogram approach (Cook and Kairiukstis 1990, Vaganov et al. 2006). Lastly iv) we produced a mean tree-ring profile per site by averaging each year and tree profiles.

3.3.7 Growth and wood-anatomy statistical models

We applied Generalized Additive Mixed Models (GAMMs, Wood 2006) to study the patterns in yearly radial increment data from dendrometers and wood cell formation from anatomical measurements. Generalized additive models are a semi parametric regression model that allows a flexible representation of the dependence of the response variable in the covariates by using a sum of smooth functions. The use of GAMMs makes it possible to model non-linear patterns in the response variable at the cost of increasing complexity in the model specification. In this case the response variable was the growth rate according to the dendrometer measurements. Growth rate was modelled as a function of two covariates: one representing the cyclic duration of the year in days (day of year), the other the duration of the experiment in days (time).

$$\text{Growth rate}_i = f_1(\text{day of year}_i) + f_2(\text{between years trend}_i) + e_i \qquad (1)$$

Where f1 and f2 represent smoothing functions of the two covariates and e_i random variable. We modelled the day of the year as a cyclic cubic regression spline in order to account for the cyclic behaviour of intra annual stem growth increments within a year and time as a cubic regression spline. In our case we used tree identity as random factor since each rate of growth represents repeated measurements from each tree. We also included an AR1 autocorrelation structure to control the cyclic variation of growth within each year.
A similar protocol was used to study the patterns in wood anatomical formation. In this case we modelled lumen area and cell wall thickness as a function of time and ring-wood sector.

$$\text{Cell size} = f_1(\text{tree-ring section}_i) + f_2(\text{time}_i) + e_i \qquad (2)$$

Each tree-ring was divided in 100 sections to represent the yearly variation in wood anatomy (tree ring section). We included tree as random factor in the analyses since measurements within each tree sample were repeated over time. We also included a correlation structure to control within tree-ring patterns in wood formation. A similar procedure was applied by Pellizzari et al. (2016). Statistical analysis, computing and graphics were performed using R (R Development Core Team, 2015).

3.4 Results

3.4.1 Radial increment analysis:

Radial-increment dynamics in Aleppo pine featured a similar bimodal pattern in both sites with two main peaks during spring and autumn and very low increment rates during summer and winter (Fig. 1). The mild-wet site at Barcelona shows higher radial increment values for both growth peaks when compared to the fluctuations observed in the continental-dry Zaragoza site. The trough is instead very similar for both sites, although it occurs earlier in Barcelona (day 205 ± 0.2, 23 July) than in Zaragoza (day 230 ± 0.2, 17 August) (t = -82.34, P <0.001) (Table 1). The growth peak in spring occurs almost simultaneously in both sites: Barcelona (133 ± 0.1, 12 May) and Zaragoza (134 ± 0.2, 13 May) (t = -5.37, P = 0.01). Instead the autumn growth season peaks slightly earlier in Zaragoza (276 ± 0.2, 2 October) than in Barcelona (280 ± 0.3, 6 October) (t = 10.48, P = 0.001).

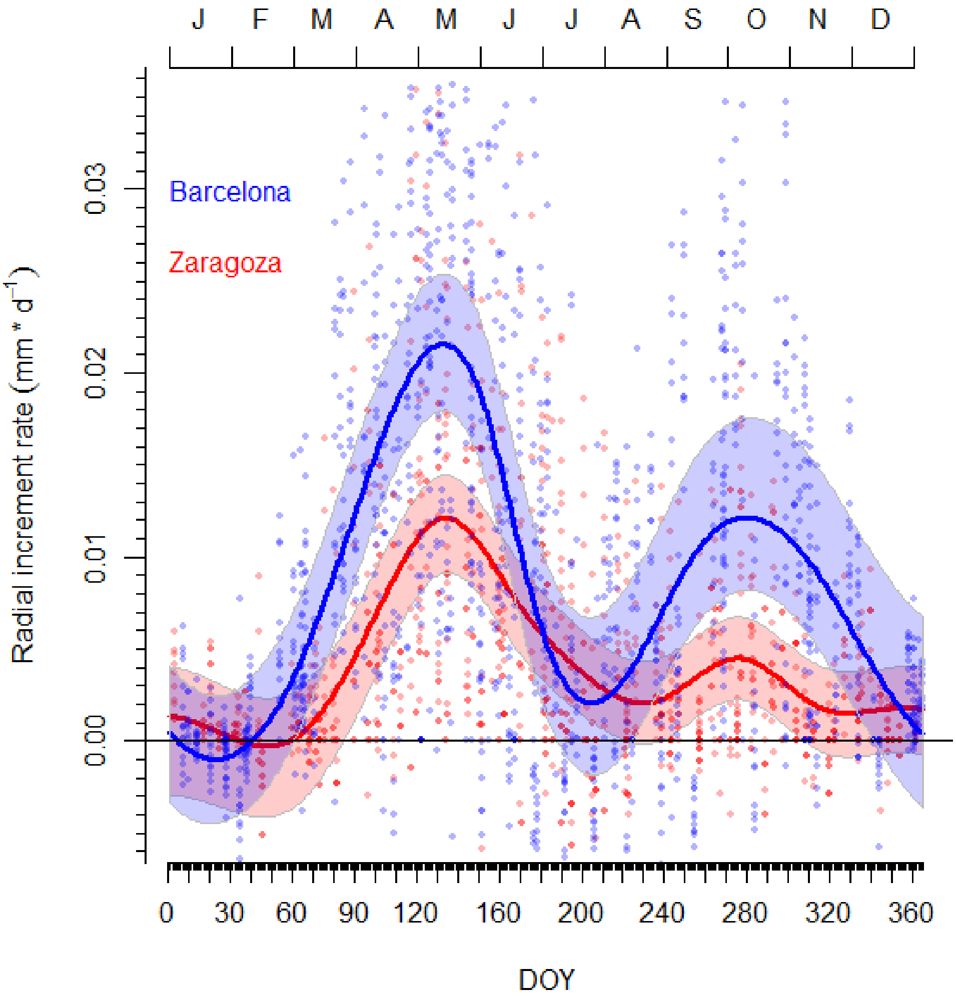

Figure 3.1 Observed (points) and fitted (lines with confidence intervals) radial increment rates for the Aleppo pines in Zaragoza (red lines and symbols) and Barcelona (blue lines and symbols). The fitted lines highlight the growth bimodality and correspond to Generalized Additive Mixed Models (GAMMs). Portrayed data corresponds to the whole measurement period on each site.

Periods of radial increment	Barcelona	Zaragoza	*t (P)*
Early peak	133 ± 0.1	134 ± 0.2	-5.37 (0.01)
Trough	205 ± 0.2	230 ± 0.2	-82.34 (<0.001)
Late peak	280 ± 0.3	276 ± 0.2	10.48 (0.001)

Table 3.1 Estimated days of the year when the early and late growth peaks and the trough between peaks are predicted to occur based on radial increment rates (dendrometer data). Estimates correspond to Aleppo pines from the Barcelona (1995-2004 period) and Zaragoza (2006-2014 period) study sites. Values are means ± SE and differences between sites were assessed using t tests. The estimated days were obtained by fitting Generalized Additive Mixed Models (GAMMs) to radial increment rates.

3.4.2 Wood anatomy features and intra-annual growth patterns:

In total, 1.29 x 10^6 and 1.25 x 10^6 tracheids were measured in the Zaragoza and Barcelona study sites, respectively. Based on these measurements we produced a very detailed quantitative description of Aleppo pine wood anatomy from 1950 to 2014 for both study sites (Fig. 2).

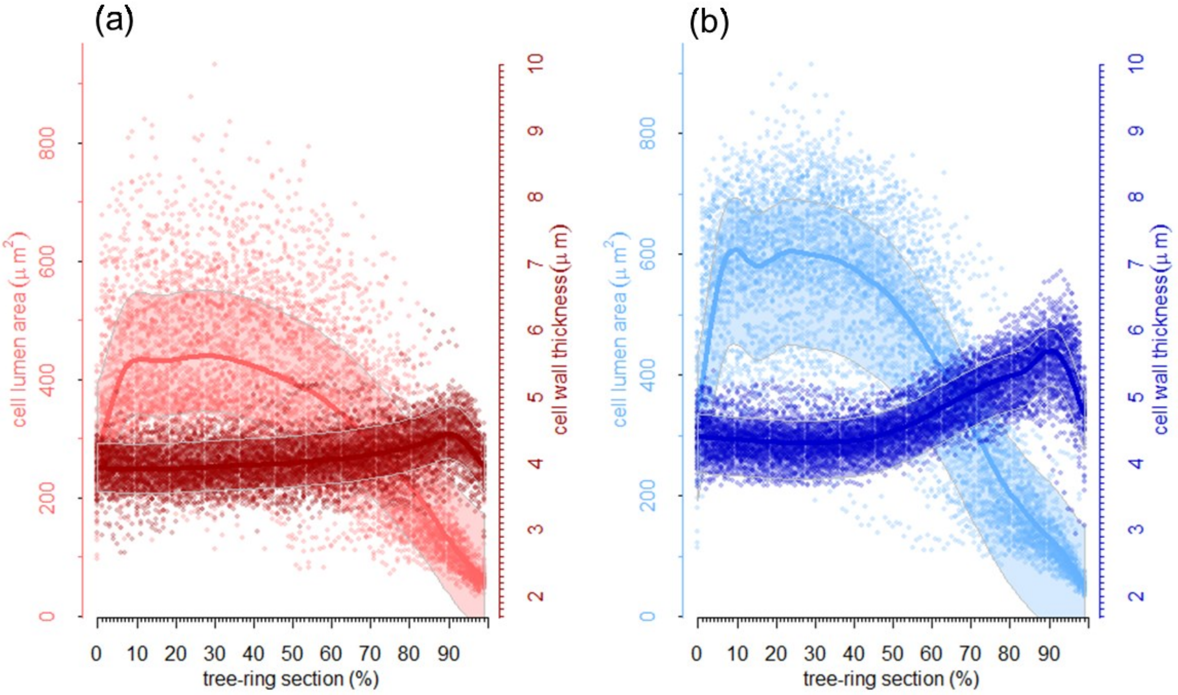

Figure 3.2 Observed (points) and fitted (lines with confidence intervals) tree-ring wood-anatomical profiles for cell lumen area (light colours) and cell wall thickness (dark colours) of Aleppo pines in Zaragoza (a) and Barcelona (b). The fits highlight the intra-annual trends in tracheid lumen area and wall thickness and correspond to Generalized Additive Mixed Models (GAMMs). Data correspond to the common 1950-2014 period.

In the continental site, tracheids showed smaller LA (333 ± 11.5 µm^2) and CWT (4.05 ± 0.01 µm) than those from the coastal region in Barcelona (424 ± 18 µm^2 and 4.7 ± 0.03 µm, respectively). The changes in cell size followed similar intra-annual trends for LA at both study sites, but with a steeper decrease in size at the coastal site when approaching the latewood. This decrease is accompanied by a strong and clear increase of CWT (with the peak reaching more than 5.5 µm), which is much more subtle in the case of the continental site in Zaragoza. The main descriptive statistics commonly used in tree-ring research were computed to compare site wood anatomy chronologies and can be consulted in supplementary material table S2 and figure S2.

The trees in Barcelona produced more IADFs than those in Zaragoza (Table 2). Barcelona trees presented 27 years with IADFs frequencies of 50% or higher, against just 20 years for the Zaragoza pines. If we consider instead the years with total absence of IADF (i.e. a 0% frequency in IADF presence), the difference between sites becomes more evident: Barcelona had just 8 years without IADF, whereas in Zaragoza there were 23 years without them.

Frequency	Barcelona	Zaragoza
≥ 50%	27	20
< 50%	36	43
100%*	8	3
0%*	8	23

*Values already accounted on the two first categories

Table 3.2 Comparison of the amount of years with intra annual density fluctuations (IADF) occurrence, considering two frequency ranges: above and below 50%, plus two absolute categories: full presence (100%) and total absence (0%), for both sites.

Selecting the years with a 50% or higher frequency of IADF (i.e. a single and clear density fluctuation per ring) it can be observed that the minimum LA within the IADF is located almost in the same sector for both sites (Zaragoza: sector 76 ± 9.2, and Barcelona: sector 74 ± 7.7), but with different characteristics (Fig. 3). While in a typical IADF year at Zaragoza the earlywood cells LA follows the main trend very closely, the Barcelona earlywood cells are well above the LA average, although still within the GAMMs confidence intervals of all the studied years. A contrasting behaviour is observed during the ring section corresponding to the IADF. The LA values of Zaragoza trees show a continuous decrease that passes below the lower limit of the GAMMs confidence intervals. Then, in the recovery phase the earlywood-like tracheids never regain average values. In the case of Barcelona, after a steep decrease in LA to reach the minimum point of the density fluctuation, it barely skirts the expected trend of the global GAMMs confidence limits, and LA values rise back to the average trend in the recovery phase.

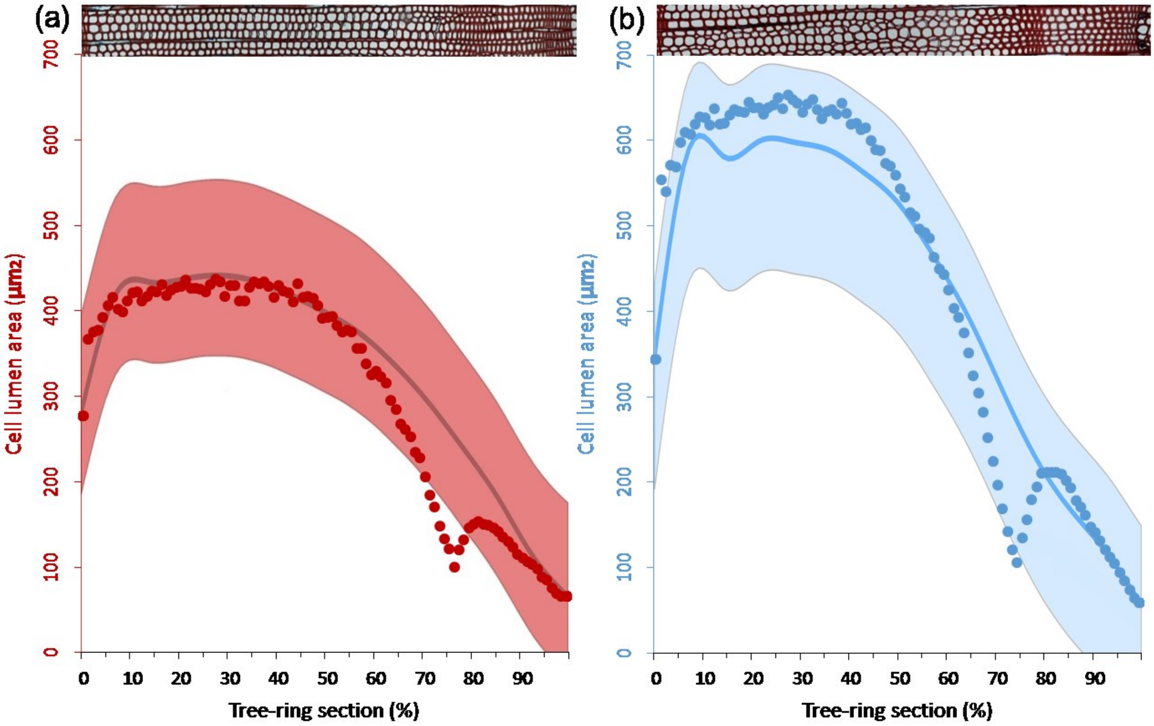

Figure 3.3 Mean tracheid lumen area profiles (red and blue dots) for the intra-annual density fluctuations (IADF) years within the 1950-2014 period of Aleppo pines from Zaragoza (a) and Barcelona (b). Shadings and lines represent the global GAMMs fitting as in Fig. 2. The top images show two typical rings with IADFs.

3.4.3 Climate-wood anatomy relationships

Considering that water availability is one of the main drivers of forest productivity and wood formation in Mediterranean sites, we calculated Pearson correlations for different weather parameters related to precipitation. The most robust and significant correlations were obtained using 40-day moving windows and accumulated soil moisture deficit (SMD). Results show clearly the importance of SMD for the enlargement of LA in tracheids of Zaragoza Aleppo pines. The significant positive correlation in Zaragoza extends throughout the growing season, even before its start for the first half of the sectors (Fig. 4), while for the second half, a clear peak of positive correlations can be observed during early summer (late June). In the case of Barcelona, only the first four sectors have a slight positive correlation mostly during late spring and early summer.

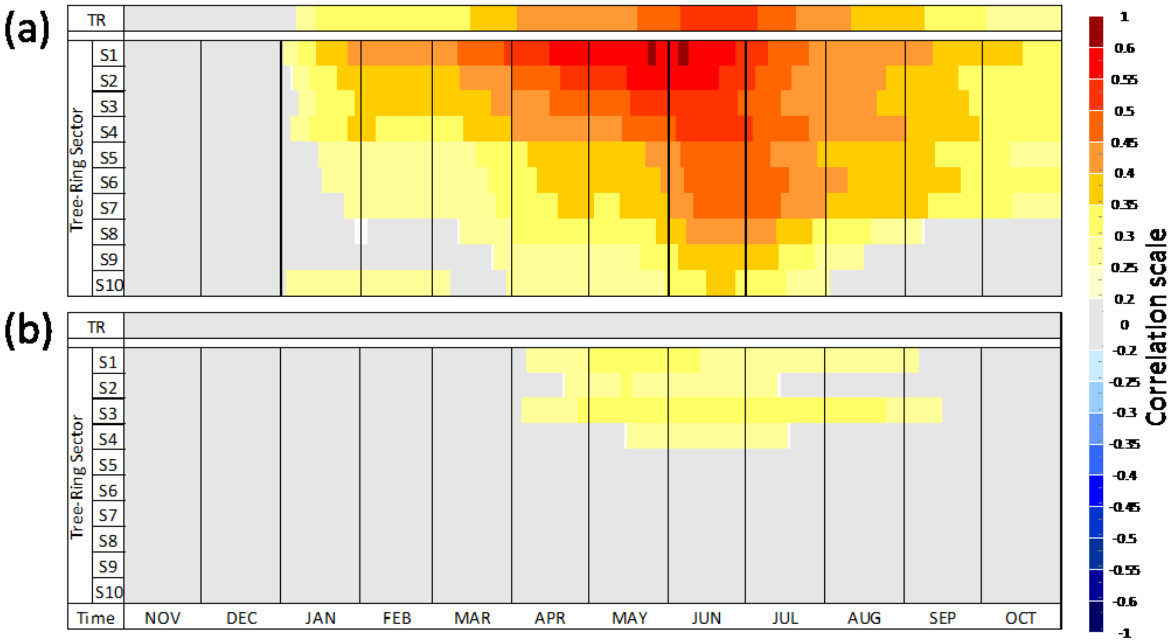

Figure 3.4 Climate-growth associations comparing lumen area with soil moisture deficit in Aleppo pine for both study sites (a, Zaragoza; b, Barcelona) and over the 1952-2012 period. Correlations were computed from November of the previous year to the following October considering each tree-ring sector separately from the earlywood (S1) to the end (S10) of the ring. Correlation coefficient above │0.254│ are significant at $P < 0.05$.

The analysis of precipitation regimes, considering the years when IADF were observed separately from those when they were absent, showed differences in both sites (Fig. 5). In Zaragoza, the years without IADFs present almost no difference from the mean average rainfall recorded during the study period (1952-2012). Instead, the IADF years have a much rainier late summer and early autumn. In addition, these IADF years have drier conditions at the beginning of the growing season (late March and early April). At the Barcelona study site the winter months (January and February) are drier than the average for IADF years, while autumn rains (beginning of September) come around 40 days earlier in IADF years than non-IADF years.

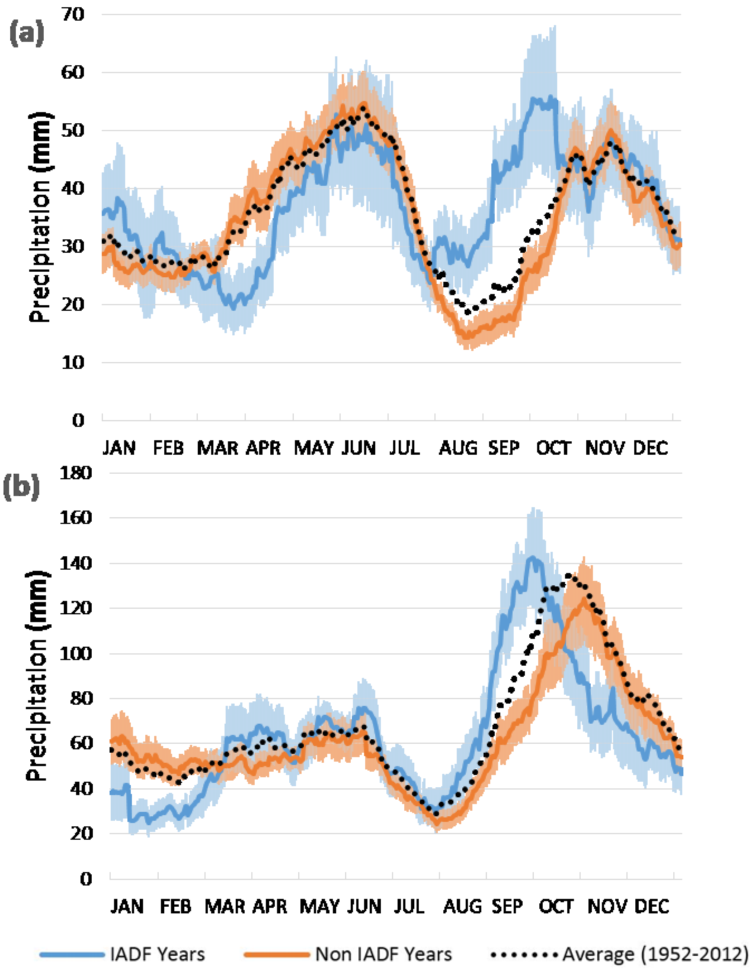

Figure 3.5 Rainfall patterns of the years with high (> 50%) and low (< 50%) frequencies of intra-annual density fluctuations (IADFs) for the two study sites (a, Zaragoza; b, Barcelona) in the 1952-2012 period. Rainfall values are calculated as 40-days running-windows; coloured bars indicate standard errors of each value.

3.5 Discussion

In this study, the combined use of high-frequency but rather short-term dendrometers records with long-term wood anatomy analysis allowed us to assess the bimodal growth of *Pinus halepensis* where the xylem anatomical traits reflect the climatic conditions driving xylogenesis. We selected Aleppo pine for its wide distribution around the Mediterranean Basin. This allowed us to compare its growth pattern between two different Mediterranean climatic regimes (coastal and continental). This comparison showed us how in wetter and milder coastal conditions the species presents a more pronounced bimodal growth, larger cells and higher frequency of IADFs than on the dry continental site.

In Mediterranean climates, many tree species have a growing season with two distinct growth peaks and a low growth activity period in between (Liphschitz and Lev-Yadun 1986). This bimodal xylogenesis behaviour is also associated with the formation of intra-annual density fluctuations (IADF) and has been reported in several conifers (*Pinus halepensis*, Ribas 2006, Camarero et al. 2010, Touchan et al. 2012, De Luis et al. 2007, 2011; *Pinus pinaster*, Rozas et al. 2011, Vieira et al. 2014, Carvalho et al. 2015; *Juniperus thurifera*, Olano et al. 2012, Pacheco et al. 2015; *Pinus pinea*, Campelo et al. 2007) and hardwood tree species (*Quercus ilex*, Gutiérrez et al. 2011; *Arbutus unedo*, Battipaglia et al. 2010, de Micco et al. 2012). Our nine and ten-year long record of radial-increment measurements portrays, in concordance with other Mediterranean conifers, a very similar bimodal pattern of growth for both sites and ages (Campelo et al. 2013, Vieira et al. 2013, 2015). The two age groups in the Barcelona site highlighted no differences on the bimodal growth pattern except for the expected distinct growth rate (Supplementary material, Figure S5). The results from the GAMMs (Fig. 1) show how this bimodality in growth rates is more consistent in Barcelona, where precipitation is higher, the weather milder and growing seasons last longer. Trees in both sites experience a similar period of low growth activity during the dry summer, but while the two peaks of high activity coincide almost perfectly in time, the trough shows a delay of almost one month in Zaragoza (Table 1). When we overlapped these periods of minimal growth activity with the average precipitation records (Fig. 5), it emerges that the trough matches with the driest days of the year for both sites (i.e. end July in Barcelona and mid-August in Zaragoza). Given that the precipitation average was calculated over a period of 60 years and matched our radial increment results, we consider that a 10-year long dendrometer record might be considered sufficient to obtain reliable stem growth data in areas with strong inter-annual weather variability.

One of the main differences between the climates at both sites is the actual amount of soil water trees may use, i.e. considering the lower rainfall and higher evapotranspiration rate in Zaragoza as compared with Barcelona, and how it is seasonally distributed. Our results on wood-anatomical traits show how Aleppo pines produce tracheids with smaller lumen area in the semi-arid Zaragoza and bigger ones in the more mesic coastal Barcelona (Fig. 2), where rainfall is twice that in the inland site (Supplementary material, Figure S1). These intrinsic climatic conditions also result in taller, even though younger, trees at the Barcelona site (Supplementary material, Table S1) which, considering the mechanistic link between conduit-lumen size, tree architecture and height growth (Carrer et al, 2015), also accounts for the difference in mean cell area between sites (Fig. 2). It is known that cambial activity in a Mediterranean climate is controlled principally by water availability during the growing season (Cherubini et al. 2003) and variations on this availability can affect the tracheids anatomical features (Pacheco et al. 2015). As mentioned before even though both sites follow the bi-seasonal rainfall pattern, Zaragoza has its highest precipitation peak during spring, while Barcelona experiences it during autumn and maintains an almost uniform precipitation from winter to the spring rainfall peak. This steady availability of water before and during the first part of the growing season explains the better overall growth and therefore bigger tracheids size in the Barcelona pines. Transversal lumen size in tracheids plays an important role in the trade-off between hydraulic conductivity and vulnerability to cavitation (Willson and Jackson 2006, Hacke 2015), and is strongly related to water availability during the phase of tracheid radial enlargement (Vaganov et al. 2006, Vieira et al. 2009, DeSoto et al. 2011). In Zaragoza the scarcity of precipitation before the growing season and the relatively lower spring rain peak (if compared with Barcelona) is probably a contributing factor to the smaller cell size of its pines.

The presence of IADF occurs mainly in the last third of the tree ring profile (Vieira et al. 2010, Rozas et al. 2011, Novak et al. 2013). Our samples show that the sharp reduction in lumen area corresponding to the IADF occurs at 0.75 of the distance from the ring border (i.e. sector 75 ±1 of the tree-ring profile), and both sites present similar minimum values of cell area (Fig. 3). This can be interpreted as trees in both sites following a similar process in the formation of summer latewood. When they enter the recovery phase (i.e. cambium reactivation), tracheids in Barcelona display lumen areas 50% bigger than Zaragoza trees, and while the first ones reach the average expected values of their predicted tree-ring profile, those from Zaragoza stay well below theirs. These long-term results complement those obtained from the dendrometer analysis, showing a stronger bimodal plasticity not just during regular years but also affecting cell production during the years with IADF formation.

Soil water availability is the environmental driver of tree growth and wood anatomy with the most striking contrast between sites. In the one hand, xeric characteristics at Zaragoza are probably the reason why lumen area positively correlates so significantly with soil moisture deficit. On the other, pines in the wetter Barcelona show a slight response to this parameter, as they grow under less limiting water conditions. Separating IADF and non-IADF years in Zaragoza, significant positive values are maintained in the non-IADF years, but they almost disappear during the years when IADFs are formed (Supplementary material, Figure S4). This may represent an indicator that IADF are formed when soil water availability is scarce, and therefore the trees show a stronger response to late-summer water availability that otherwise would not trigger the formation of an earlywood-like IADF. As seen in Figure 5, the years with IADF formation registered a significant drop in precipitation a couple of weeks before and during spring cambial activation (i.e. late March for Zaragoza and February in Barcelona). This condition, together with the precipitations in late summer and early autumn, is a clear example of the complexity of factors contributing to wood formation and its intra-annual features.

3.6 Conclusions

In conclusion, we found that growth bimodality and IADF formation, two facets of the double period of radial growth and wood formation in Mediterranean forests, were more pronounced in the mild-wet site (Barcelona) than in the cold-dry site (Zaragoza), which also displayed a shorter growing season. In the drier site, tracheid lumen area responded to short-term (weekly scales) changes in precipitation and soil water availability during the period when growth rates peaked, i.e. late spring (May) to early summer (June) (compare Figs. 1 and 4). Furthermore, trees in the wetter Barcelona site formed wider lumen areas, which in addition to the longer growing season, could explain why they also presented higher growth rates, more IADFs and a more pronounced bimodal behavior.

Matching short-term intensive monitoring with long-term retrospective wood anatomy records proved to be an efficient combination for a quantitative and robust assessment of cambial phenology (Carrer et al. 2017), IADFs (Battipaglia et al., 2016) and to define the climate drivers triggering the bimodal growth pattern in Mediterranean environment.

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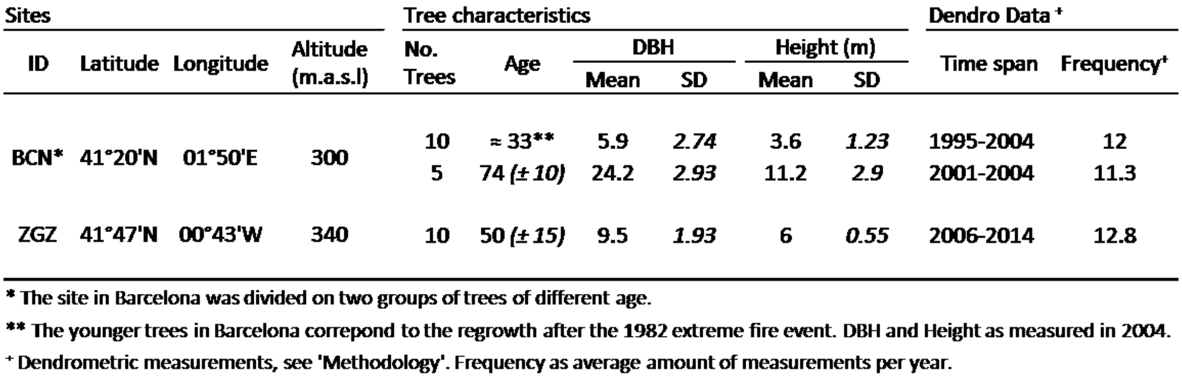
3.8 Supplementary Material



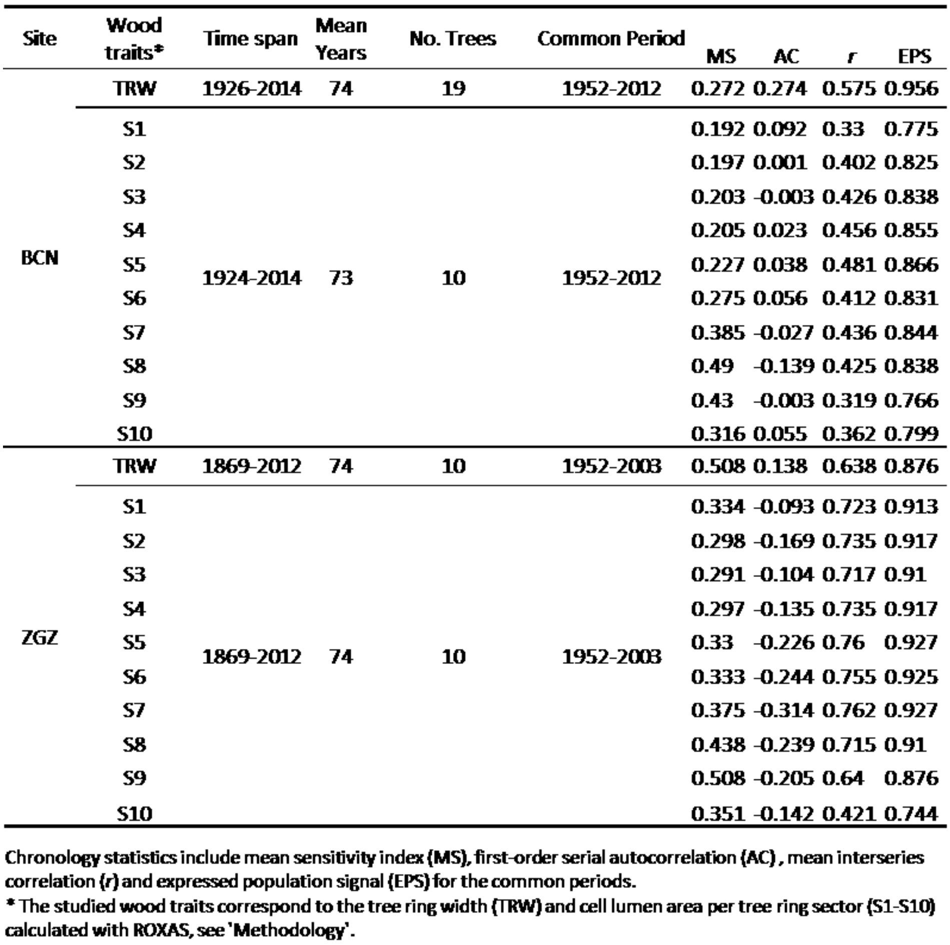
Sites				Tree characteristics						Dendro Data ⁺	
ID	Latitude	Longitude	Altitude (m.a.s.l)	No. Trees	Age	DBH		Height (m)		Time span	Frequency⁺
						Mean	SD	Mean	SD		
BCN*	41°20'N	01°50'E	300	10	≈ 33**	5.9	*2.74*	3.6	*1.23*	1995-2004	12
				5	74 *(± 10)*	24.2	*2.93*	11.2	*2.9*	2001-2004	11.3
ZGZ	41°47'N	00°43'W	340	10	50 *(± 15)*	9.5	*1.93*	6	*0.55*	2006-2014	12.8

* The site in Barcelona was divided on two groups of trees of different age.

** The younger trees in Barcelona correpond to the regrowth after the 1982 extreme fire event. DBH and Height as measured in 2004.

⁺ Dendrometric measurements, see 'Methodology'. Frequency as average amount of measurements per year.

Table S 3.1. Geographical features and tree characteristics of the study sites where the dendrometric measurements were conducted.



Site	Wood traits*	Time span	Mean Years	No. Trees	Common Period	MS	AC	*r*	EPS
BCN	TRW	1926-2014	74	19	1952-2012	0.272	0.274	0.575	0.956
	S1	1924-2014	73	10	1952-2012	0.192	0.092	0.33	0.775
	S2					0.197	0.001	0.402	0.825
	S3					0.203	-0.003	0.426	0.838
	S4					0.205	0.023	0.456	0.855
	S5					0.227	0.038	0.481	0.866
	S6					0.275	0.056	0.412	0.831
	S7					0.385	-0.027	0.436	0.844
	S8					0.49	-0.139	0.425	0.838
	S9					0.43	-0.003	0.319	0.766
	S10					0.316	0.055	0.362	0.799
ZGZ	TRW	1869-2012	74	10	1952-2003	0.508	0.138	0.638	0.876
	S1	1869-2012	74	10	1952-2003	0.334	-0.093	0.723	0.913
	S2					0.298	-0.169	0.735	0.917
	S3					0.291	-0.104	0.717	0.91
	S4					0.297	-0.135	0.735	0.917
	S5					0.33	-0.226	0.76	0.927
	S6					0.333	-0.244	0.755	0.925
	S7					0.375	-0.314	0.762	0.927
	S8					0.438	-0.239	0.715	0.91
	S9					0.508	-0.205	0.64	0.876
	S10					0.351	-0.142	0.421	0.744

Chronology statistics include mean sensitivity index (MS), first-order serial autocorrelation (AC) , mean interseries correlation (*r*) and expressed population signal (EPS) for the common periods.

* The studied wood traits correspond to the tree ring width (TRW) and cell lumen area per tree ring sector (S1-S10) calculated with ROXAS, see 'Methodology'.

Table S 3.2 Descriptive statistics for Aleppo pine (*Pinus halepensis*) tree-ring and anatomical chronologies in both study sites.

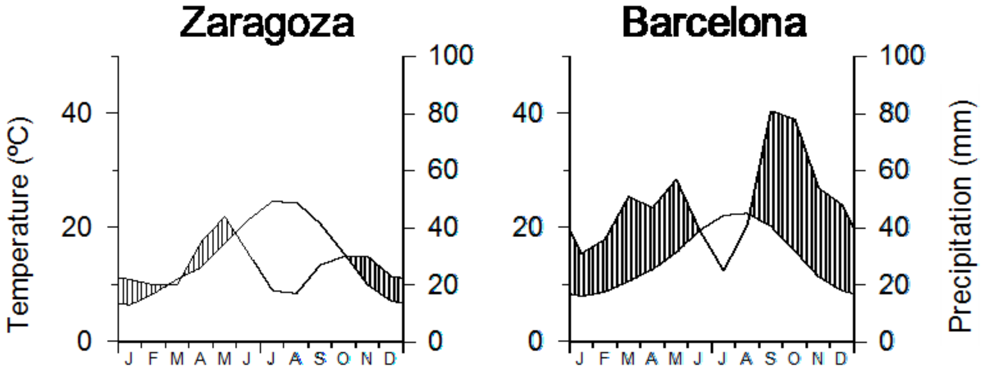


Figure S 3.1. Climate diagrams of the weather stations situated near the two study sites with data for the period 1950-2014.

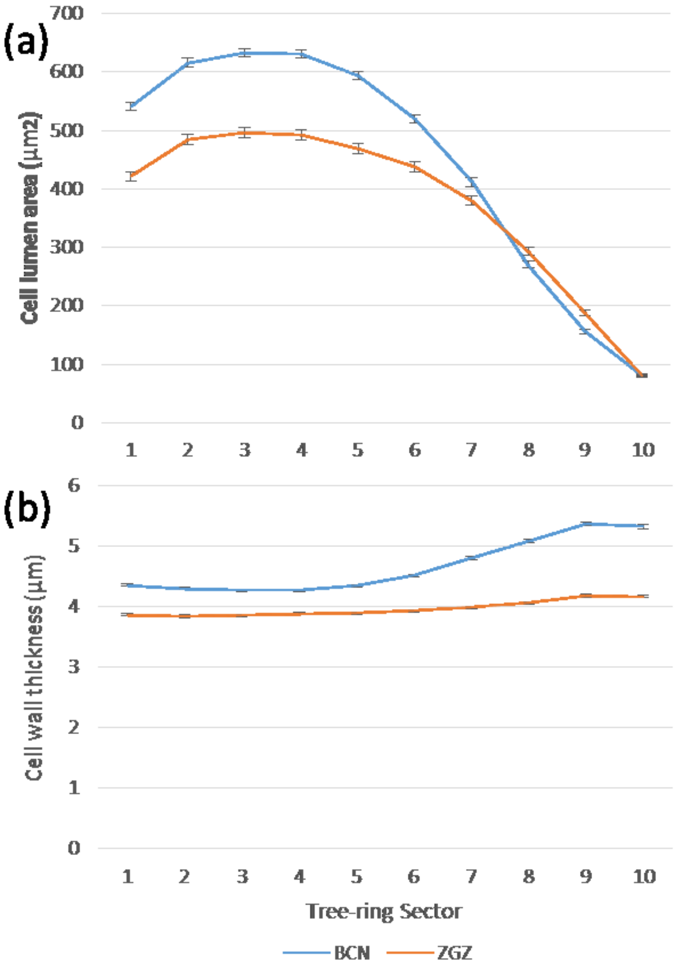


Figure S 3.2 Mean values of lumen area (a) and cell wall thickness (B) calculated per tree-ring considering all trees and the whole study period (1950-2014) for the two study sites (Zaragoza and Barcelona). Bars indicate standard errors of each value.

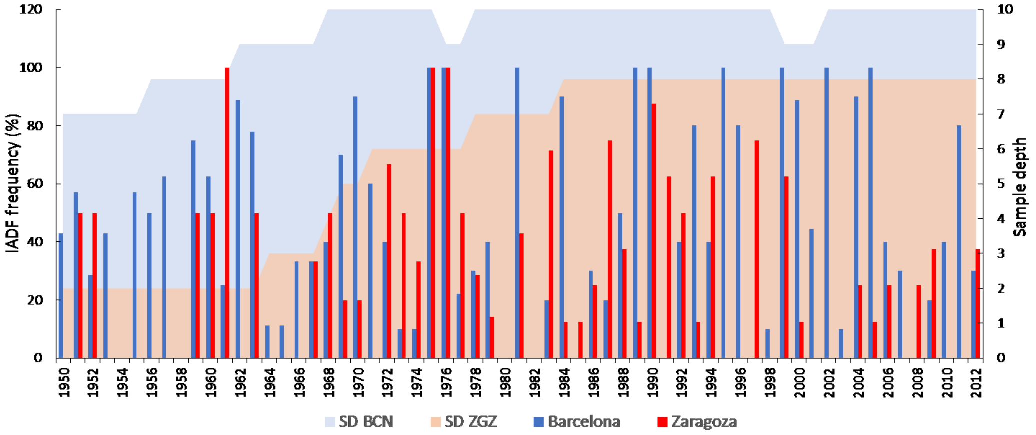


Figure S 3.3. Intra-annual density fluctuation (IADF) frequencies for the whole study period (1950 – 2012) of Barcelona (blue columns) and Zaragoza samples (red columns). Shaded background areas show the sample depth per year for each site.

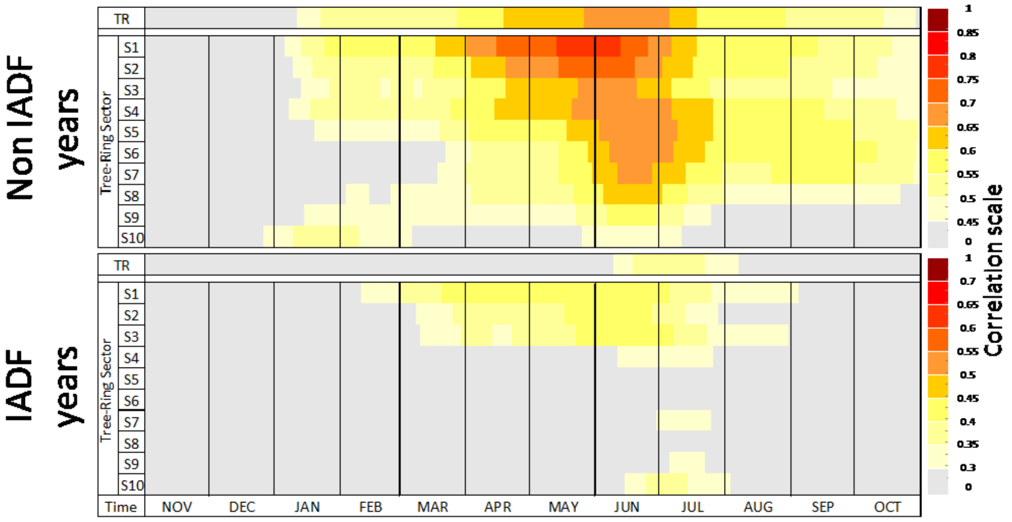


Figure S 3.4. Climate-growth associations comparing lumen area with soil moisture deficit in Aleppo pine from the Zaragoza study site. A 60-year period (1952-2012) has been divided in two categories according to the presence of intra-annual density fluctuations (IADF): IADF (with +50% trees presenting IADFs) and non-IADF (with -50% trees presenting IADFs) years. Correlations were calculated annually from November of the previous year to the following October (x axes) against the lumen area values of each tree ring (TR) and each of its 10 constituting sectors along the radial direction from the beginning (S1) to the end (S10) of the ring (left y axes). Correlations coefficient above |0.305| for non-IADF years and |0.456| for IADF years are significant at $P < 0.05$.

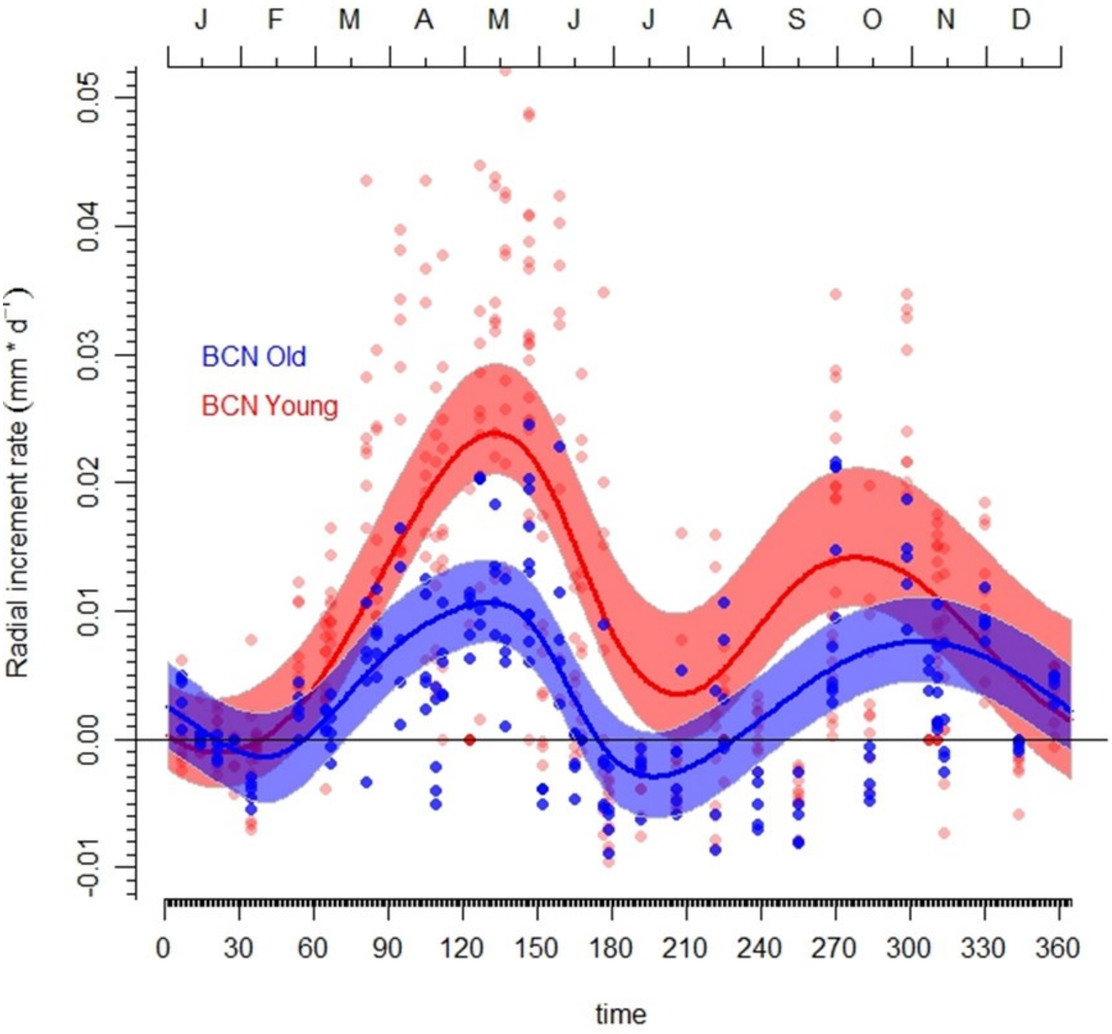


Figure S 3.5 Observed (points) and fitted (lines with confidence intervals) radial increment rates for Aleppo pine in Barcelona, comparing young trees (red lines and symbols) with old ones (blue lines and symbols). The fitted lines highlight the growth bimodality and correspond to Generalized Additive Mixed Models (GAMMs). Portrayed data corresponds to the whole measurement period.

4. Shifts of irrigation in Aleppo pine under semi-arid conditions reveal uncoupled growth and carbon storage and legacy effects on wood anatomy

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Keywords: drought, irrigation, dendroanatomy, *Pinus halepensis*, non-structural carbohydrates, wood parenchyma, xylem.

4.1 Abstract

Climate models forecast progressive aridification in the Mediterranean region. These warmer and drier conditions will probably lead to a decrease in carbon fixation and growth rates of drought-prone forests. To improve our knowledge on how water availability drives radial growth, wood anatomy and carbon storage we compared changes during a 40-year period in tree-ring width, wood-anatomical traits (tracheid lumen area, cell-wall thickness and parenchyma area), and stem sapwood non-structural carbohydrates (NSC) concentrations, in coexisting trees subjected to different irrigation regimes. We studied the drought-tolerant Aleppo pine (*Pinus halepensis*) comparing trees subjected to three irrigation regimes (control trees, no irrigation; IR trees, irrigated trees; TI trees, temporarily irrigated trees). Tree-ring width of TI trees widely overtook growth rates shown by IR trees during the irrigation treatment (1980s), whereas transversal lumen area and cell-wall thickness presented similar values in both sets of trees. After the irrigation period, TI and control trees showed similar year-to-year growth variability. Lumen area of TI trees took 4 to 5 years to show values similar to those of the non-irrigated group, indicating that long-term irrigation has legacy effects on wood anatomy. The TI trees had lower ray parenchyma area percentages during the irrigation period; afterwards both TI and non-irrigated trees formed similar amounts of ray parenchyma which were always higher than in IR trees. NSC concentrations were lower for IR trees suggesting either a dilution effect due to the significantly larger biomass of irrigated trees or a higher demand for NSC by other carbon sinks such as primary meristems. Growth and xylem anatomy results indicate that the drought-tolerant Aleppo pine is able to coordinate carbon supply and demand (growth) under changing conditions of water availability.

4.2 Introduction

Drought is one of the major constraints to forest growth and productivity and is expected to become increasingly crucial in many regions because of the ongoing climate warming (Vicente-Serrano et al. 2013). Recent droughts have severely impacted forests worldwide causing: die-off events and tree mortality (Allen et al. 2010, Camarero et al. 2015), transient declines in forest productivity (Baldocchi 1997, Ciais et al. 2005, Dorman et al. 2013, Leuzinger et al. 2005) and higher vulnerability to attacks by biotic agents (Bréda et al. 2006). Improving our knowledge on the factors controlling tree growth is thus a critical step to assess and predict the impact of a drier climate on forests. This is specially compelling for Mediterranean areas where climate models predict warming and aridification (IPCC 2013), which may threaten the survival of some pine stands and tree species at very xeric sites (Sánchez-Salguero et al. 2017, 2012).

There is evidence supporting drought-induced sink limitation in trees because low water availability restricts carbon sinks (growth) more than sources (photosynthesis) (Lempereur et al. 2015), leading to an accumulation of non-structural carbohydrates (NSC hereafter) and often uncoupling carbon use (growth) from carbon uptake (photosynthesis) (Körner 2003, Muller et al. 2011). Consequently, drought-triggered growth reduction can lead to increases in the concentrations of NSC (Sala and Hoch 2009). However, NSC accumulation may also be explained by an active growth suppression to avoid carbon starvation under prolonged water shortage (McDowell 2011, Wiley and Helliker 2012). In that case, carbon starvation could be adaptive and driven by other factors such as phloem malfunction (Sala et al. 2012, Sevanto 2014). This agrees with the observations that drought-tolerant tree species increase NSC content in response to water shortage (Piper 2011) or with the conversion of stored NSC (e.g. starch) into mobile osmolytes (e.g. soluble sugars) during drought (Salleo et al. 2009). Such controversial views on how water availability drives growth dynamics and NSC storage require further insight (Hartmann and Trumbore 2016). In addition, improved knowledge on growth-NSC relationships will be helpful in forecasting the responses of Mediterranean forests to predicted aridification since droughts have already severely impacted these forests in the late 20th century causing declines in forest productivity (Sarris et al. 2007, Dorman et al. 2013) and triggering die-off and mortality events (Camarero et al. 2015).

Aleppo pine (*Pinus halepensis* Mill.) is one of the most common conifers in the Mediterranean Basin and presents a high growth plasticity and drought tolerance in response to changing water availability, which makes it an ideal subject for studying the effects of drought on tree performance in this region (Girard et al. 2012, Gazol et al. 2017). According to Borghetti et al. (1998) mature Aleppo pines subjected to long-term drought (≥ 12 months) showed significant reduction in most growth parameters followed by a quick recovery of the overall plant hydraulic resistance a few months after the drought treatment. The low wood density in Aleppo pine roots and its vulnerability to xylem embolism has been proposed as a way to protect needles or twigs more exposed to xylem cavitation (Oliveras et al. 2003). At stem level, tracheid lumen area in earlywood can be up to 30% smaller in trees growing in dry areas which allow them to better cope with severe droughts (Pacheco et al. 2017). These strategies for withstanding drought may lead trees to adjust their water conducting tissues to a more negative hydraulic pressure (Bréda et al. 2006). This is a cumulative long-lasting process, as the sapwood consists of multiple annual rings, and such long-term adjustments can be analyzed retrospectively combining growth responses and changes in wood anatomical traits that are proxies of hydraulic functioning and resilience to drought (Montwé et al. 2014).

Drought effects on growth and NSC have mostly been studied on saplings or seedlings under imposed drought treatments or different irrigation regimes, indicating a reduction in stem biomass and growth rate, an accumulation of shoot starch in drought-exposed individuals, and the development of carbon starvation under extreme drought conditions (Villar-Salvador et al. 1999, de Luis et al. 2011, Klein et al. 2011). However, there is a lack of similar approaches on mature trees under field conditions.

In this study, we analyze three groups of mature Aleppo pine trees growing in semi-arid conditions at the same site. Throughout 40 years one group was permanently irrigated, another was irrigated only during a 10-year period, and the third was growing without any additional watering. Following this experimental design, the objective of our research is to describe and quantify the long-term effects of irrigation on radial growth, wood anatomical traits (lumen area, cell wall thickness, and parenchyma area percentage) and NSC storage in the sapwood. We hypothesize that irrigation will cause an uncoupling between growth, wood anatomy and NSC storage leading to the formation of: wide rings, tracheids with ample lumen areas and thick walls, wood with less parenchyma area and sapwood with lower NSC concentrations. Consequently, we expect that drought would lead to the opposite features.

4.3 Materials and methods

4.3.1 Tree species, study area and climate

Aleppo pine (*Pinus halepensis* Mill.) is a drought-tolerant evergreen conifer species, widely distributed across the Western Mediterranean Basin, and covering areas subjected to seasonal water deficit, mainly lowlands under dry to semi-arid conditions (Ne'eman and Trabaud 2000). Our study site is an Aleppo pine plantation located in the southeastern sub-urban area of Zaragoza (north-eastern Spain) at 340 m a.s.l. and on an almost flat topography (Table 1). Two of the sites (irrigated and non-irrigated trees) are contiguous, while the temporarily irrigated plot is 3 km to the west. This last plot was irrigated from 1980 to 1989. Mean density and basal area are 352 stems ha^{-1} and 12 m^{2} ha^{-1}, respectively. The understory vegetation is dominated by shrubs (*Genista scorpius*, *Thymus vulgaris* and *Rosmarinus officinalis*). The soils are cambisols poorly developed over gypsum outcrops. More details on sampling sites are available in Sangüesa-Barreda et al. (2015).

According to data from the nearby Zaragoza-airport weather station (41° 40' N, 1° 00' W, 263 m a.s.l.), 10 km from the study sites, the climate in the area is Mediterranean of semi-arid type. During the 1950-2012 period, mean annual temperature was 14.8 ºC (the coldest and warmest months were January and July, respectively) and total annual precipitation was 325 mm (the wettest and driest months were May and July, respectively) (Supplementary material, Fig. S1).

Table 4.1 Geographical features and characterization of the sampled *Pinus halepensis* trees from the control (non-irrigated trees) and two treatments (IR: irrigated plot; TI: temporarily irrigated plot). DBH is diameter at breast height (measured at 1.3 m). Values are means ± SE.

Site characteristics			Tree characteristics				
Latitude	Longitude	Altitude (m a.s.l)	Plots	No. Trees	Age at 1.3 m (years)	DBH (cm)	Height (m)
41° 47' N	00° 43' W	340	IR	10	48 ± 4	46.3 ± 2.5	13.3 ± 0.2
			Control	10	55 ± 7	16.4 ± 0.8	6.3 ± 0.2
			TI	10	51 ± 4	35.9 ± 1.2	14.1 ± 0.4

4.3.2 Field sampling and tree-ring data

During the winter of 2016, a total of 30 dominant and mature trees were sampled (10 per class considering control, IR and TI trees). We took two radial cores per tree at 1.3 m with 5-mm Pressler increment borers. Sampled trees were randomly distributed across the plantation and field sampling followed standard dendrochronological methods (Fritts 2001). Wood samples were air-dried and polished with a series of successively finer grits sand-paper until rings were clearly visible. Tree rings were measured to the nearest 0.01 mm using a binocular scope and a LINTAB measuring device (Rinntech, Heidelberg, Germany). Cross-dating of the tree rings was checked using the COFECHA program (Holmes 1983). Tree age at 1.3 m was determined by counting rings in the oldest core. With off-center cores we used a geometric pith locator to estimate the number of missing rings (Duncan 1989).

4.3.3 Wood-anatomy analyses

A third 10-mm core was obtained from each tree for wood-anatomy analyses. In the laboratory, these cores were divided into 4-cm long pieces, boiled in water to soften the wood and remove the resin, and sliced into 10-12 μm thick transversal sections using a rotary microtome (Leica RM 2025, Heidelberg, Germany). The samples were then stained using a water-diluted mixture of safranin (1%) and astrablue (0.5%), and rinsed with water and ethanol. Afterwards sections were fixed on permanent slides with Eukitt (BiOptica, Milan, Italy)and scanned using a D-sight 2.0 System (Menarini Diagnostics, Florence, Italy) at 100X magnification, with a resolution of 1.99 pixels μm^{-1}. The images were then processed with the image analysis software ROXAS v3 (von Arx and Carrer 2014, Prendin et al. 2017) that provided the lumen area (LA), cell-wall thickness (CWT), and relative position within the dated annual ring for each of the > 3.5 million measured tracheids. Tree-ring borders were manually drawn on the images and cross-dating was checked using the reference curves obtained in the previous tree-ring measurements. Information related to cell position was used to better define the intra-annual profile of LA and CWT. For this, rings were divided in ten sectors following Castagneri et al. (2017). Lastly, we performed a supplementary manual analysis of the images with ROXAS to extract the values of the percentage area of transversal ray parenchyma in three separate years per decade (1972, 1975, 1978…, 2002, 2005 and 2008). The analysis was performed on the rings corresponding to the period from 1970 to 2015 (Supplementary material, Fig. S2).

4.3.4 Non-structural carbohydrates in stem sapwood

We quantified the mass-based concentrations of non-structural carbohydrates (NSC) in stem sapwood to compare the carbon status of trees subjected to different irrigation regimes (Hoch et al. 2003, Hoch 2015). We selected ten individuals in each of the three pine classes (control, IR and TI trees). Sampling was conducted in February 2016 before growth started. In each individual, we sampled the stem sapwood by taking two radial cores at 0.5 m using a Pressler increment borer. All samples were collected between 10 and 12 a.m. Samples were transported to the laboratory in a portable cooler and then dried in an oven for 72 h at 60 °C. The outer sapwood portion (ca. 5 cm) was visually identified and separated from each core using a razor blade. In the case of TI trees, we considered sapwood rings corresponding to the irrigation period during the 1980s and those corresponding to the post-irrigation period (1990-2010) separately. Wood samples were subsequently frozen and stored at -20 °C until freeze dried. All dried samples were weighed and milled to a fine powder in a ball mill (Retsch Mixer MM301, Leeds, UK). Soluble sugars (SS) were extracted with 80% (v/v) ethanol and their concentration determined colorimetrically using the phenol-sulfuric method (Buysse and Merckx 1993). Starch and complex sugars remaining after ethanol extractions were reduced enzymatically. NSC measured after ethanol extraction are referred to as SS (soluble sugars) and carbohydrates measured after enzymatic digestion are referred to as starch. The sum of SS and starch are NSC.

4.4 Results

4.4.1 Growth and wood anatomical features:

Tree-ring width was significantly and constantly lower in the non-irrigated trees (control plot, mean 0.6 mm) if compared to the irrigated trees (IR plot, mean 3.2 mm) during most of the common period from 1970 to 2010 (Fig. 1). During the 1980s, when irrigation was provided to the temporarily irrigated trees (TI plot), their mean ring-width almost doubled (mean 5.7 mm) that of the IR trees. From 1990 onwards, with no more irrigation, growth of TI trees decreased to values similar to those of the control plot, being slightly wider (2.1 mm) but not significantly different compared to control trees (0.4 mm).

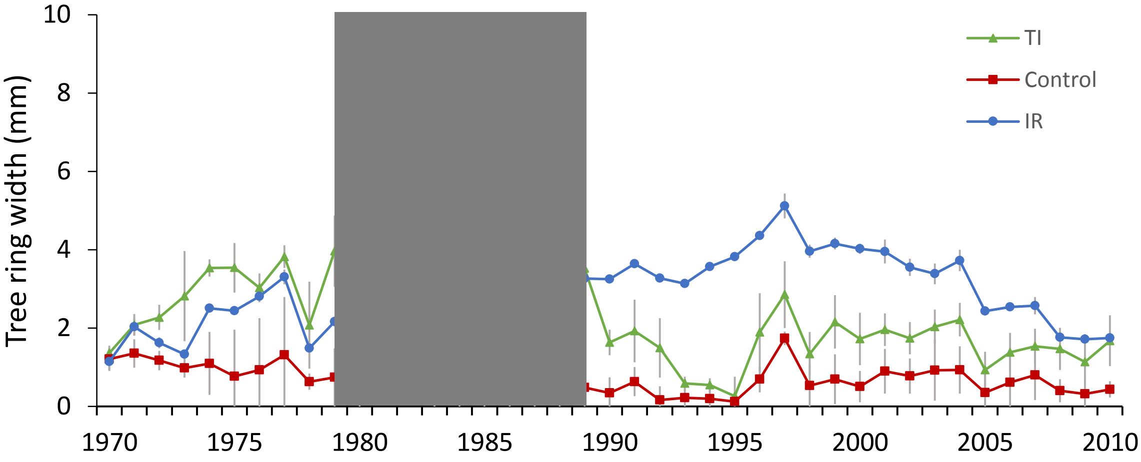

Figure 4.1 Tree ring width (means ± SE) of the three Aleppo pine treatments (TI: temporarily irrigated trees; control: non-irrigated trees; IR: irrigated trees). Data correspond to the common period of 1970-2010. Shadowed area indicates temporary irrigation period.

We produced a very detailed quantitative description of the anatomical traits for each of the study plots by measuring 0.95 x 10^6, 1.32 x 10^6 and 1.38 x 10^6 tracheids for the control, IR, and TI trees, respectively (Fig. 2). Non-irrigated trees showed smaller tracheid dimensions derived from both reduced lumen area (LA = 445.7 ± 24.1 □m^2) and cell wall thickness (CWT = 4.66 ± 0.08 □m) when compared with the other two plots. While the IR trees showed significantly higher values for both wood traits (LA = 724.3 ± 27.1 □m^2; CWT = 5.88 ± 0.11□□m), the TI trees presented intermediate values (LA = 613.9 ± 34.2 □m^2; CWT = 5.54 ± 0.12 □m). In the case of LA, TI and IR trees showed similar inter-annual variability since the 1990s. This last result mirrors year-to-year changes in tree-ring width presented in Figure 1.

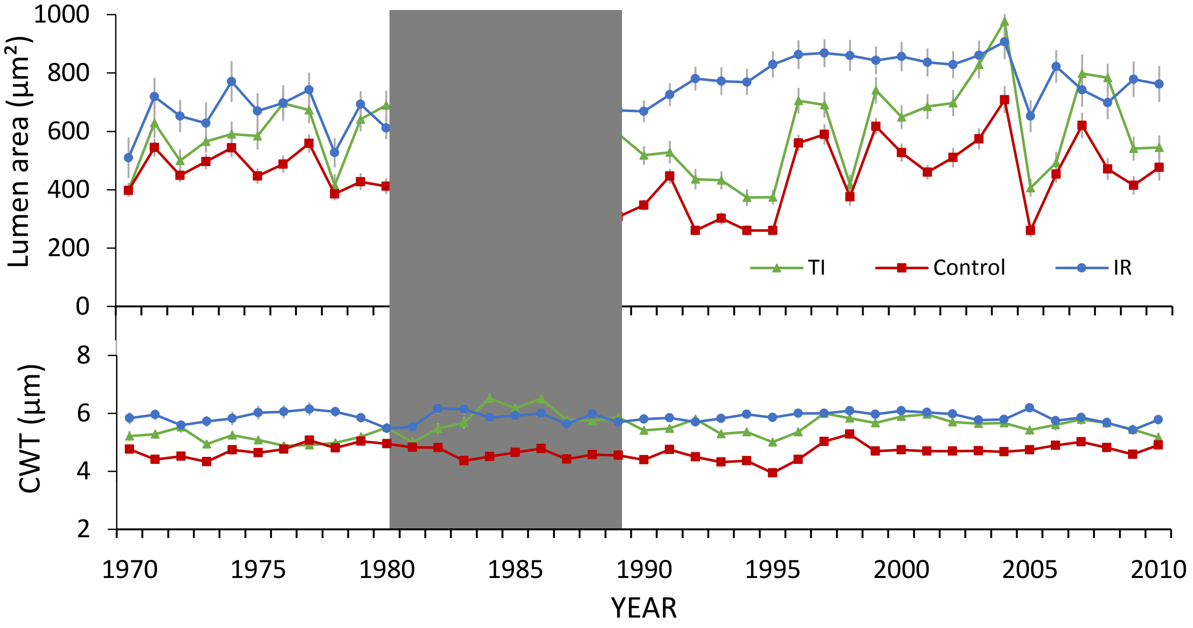

Figure 4.2 Wood anatomy series (means ± SE) of lumen area and cell wall thickness (CWT) for the common period of 1970-2010 in the three Aleppo pine treatments (TI: temporarily irrigated trees; control: non-irrigated trees; IR: irrigated trees). Shadowed area indicates irrigation period in the TI plot.

4.4.2 Intra-annual growth patterns:

During the 1980s, IR and TI trees showed no significant differences in their intra-annual wood-anatomy profiles (Fig. 3). During the other decades, tracheid lumens were wider in IR trees but smaller in control trees. This difference magnified in warm and dry periods such as the early 1990s when radial growth was very low (0.1 – 2.0 mm) in control and TI trees (Fig. 1). CWT values did not differ between TI and IR trees during the 1980s, but differences appeared in the other decades and particularly in the latewood (6th-10th sectors). Sector by sector values are available for consultation in Table S1, Supplementary material.

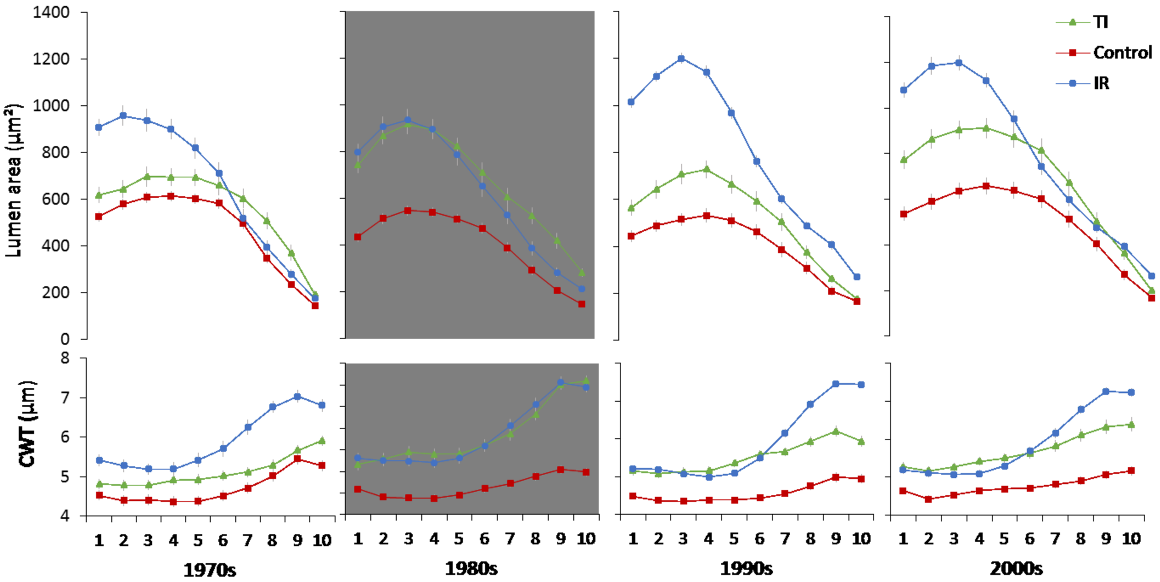

Figure 4.3 Intra-annual values (means ± SE) of lumen area and cell wall thickness (CWT) of the three Aleppo pine treatments (TI: temporarily irrigated trees; control: non-irrigated trees; IR: irrigated trees). Values are shown separately for each decade of the common period (1970-2010). The shadowed area indicates the decade under temporary irrigation.

4.4.2 Sapwood non-structural carbohydrate and ray parenchyma

Only the IR trees showed a significantly lower percentage of non-structural carbohydrates (NSC), especially the starch component (Fig. 4). Soluble sugar concentrations showed no differences among the treatments.

Analyses of the NSC of TI trees in rings formed during the irrigation period (1980s) and post-irrigation period (1990-2010) resulted in non-significant differences ($p = 0.028$) showing NSC concentrations of 3.27 ± 0.13% and 3.37 ± 0.13%, respectively. In contrast, and considering the 1980 – 2010 period, the average values of wood anatomical traits were significantly lower for control trees, while tree-ring width values differed between all treatments (Table 2).

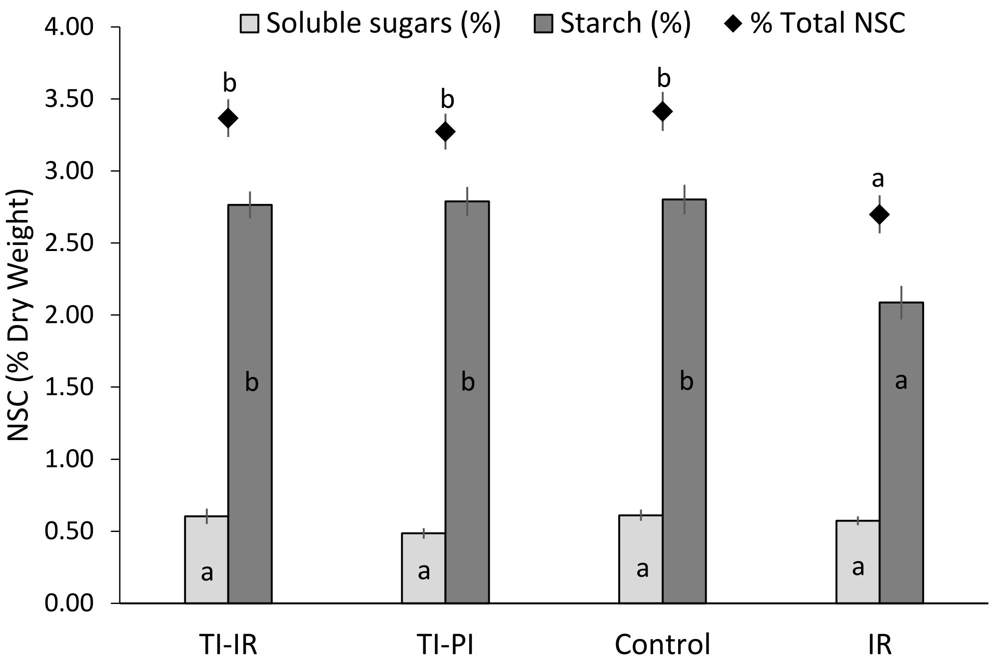

Figure 4.4 Sapwood non-structural carbohydrate (NSC) concentrations obtained in TI-IP (temporarily irrigated trees, tree rings formed during the irrigation period in the 1980s), TI-PI (temporarily irrigated trees and rings corresponding to the post-irrigation period, 1990-2010), control (non-irrigated trees) and IR (irrigated trees). NSC was divided into starch and soluble sugars represented as bars with different fills, while black diamonds are the summed amount of all NSC. Error bars show standard errors and different letters represent significant (*p* < 0.05) differences between groups according to Mann-Whitney *U* tests.

Table 4.2 Mean growth (tree-ring width), wood-anatomical traits (lumen area, cell-wall thickness) and stem sapwood non-structural carbohydrate (NSC) concentrations in Aleppo pine trees. Trees are classified as irrigated (IR), non-irrigated (control), and temporarily irrigated (TI) trees. Rings of TI tree are analyzed separately for the irrigation- (TI-IP) and post-irrigation (TI-PI) periods. Growth and anatomy data correspond to the common period 1980-2010. NSC values are shown considering soluble sugars (SS), starch and total NSC concentrations. Different letters indicate significant (*p* < 0.05) differences according to Mann-Whitney *U* tests. Values are means ± SE.

	Tree-ring width (mm)	Wood anatomy		Sapwood NSC concentration		
		Lumen area (µm²)	Cell-wall thickness (µm)	SS (%)	Starch (%)	Total NSC (%)
IR	3.23 ± 0.13	750.2 ± 28.5 b	5.88 ± 0.08 b	0.57 ± 0.03 a	2.09 ± 0.12 a	2.70 ± 0.13 a
Control	0.57 ± 0.04	435.8 ± 19.0 a	4.66 ± 0.05 a	0.61 ± 0.04 a	2.80 ± 0.10 b	3.41 ± 0.14 b
TI-IP	5.78 ± 0.42	678.4 ± 45.6 b	5.86 ± 0.16 b	0.48 ± 0.04 a	2.79 ± 0.10 b	3.27 ± 0.13 b
TI-PI	1.55 ± 0.14	598.8 ± 32.4 b	5.58 ± 0.09 b	0.60 ± 0.05 a	2.76 ± 0.09 b	3.37 ± 0.13 b

Overall, IR trees formed significantly ($p < 0.05$) less ray parenchyma area (3.9% on average) than control (5.2%) and TI (5.4%) trees, but differences between the two last groups were not significant for most of the years. IR and TI trees had similar percentages of ray parenchyma area during the 1980s, after which IR trees featured a rather constant percentage (ca. 4%) while TI trees showed an increase in ray area which stayed close to that of the control group (ca. 6%). It is possible to appreciate a recent convergence in percentage ray area (4-5%) among the three groups of trees, likely in response to wet conditions in 1988, 2002 and 2008 (Fig. 5).

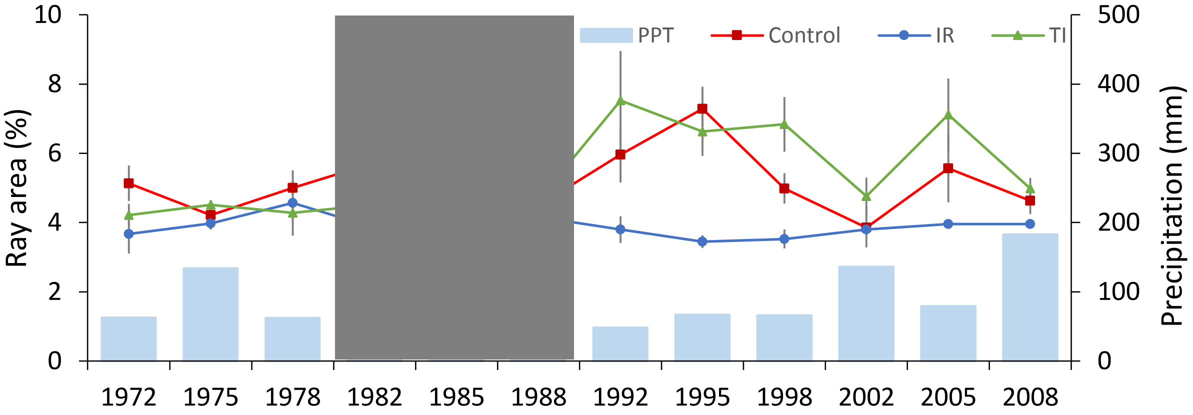

Figure 4.5 Percentage of parenchyma ray area for the three Aleppo pine groups (TI: temporarily irrigated trees; control: non-irrigated trees; IR: irrigated trees) measured from transversal anatomical sections. Values are means ± SE for each depicted year and bars show the precipitation sum from March to June of each year. Shadowed area indicates temporary irrigation during the 1980s.

4.5 Discussion

As hypothesized, irrigation enhanced height and radial growth (increasing ring width; Fig. 1), allowed the formation of tracheids with wider lumen areas and thicker cell walls (Figs. 2 and 3), reduced the sapwood NSC concentration (Fig. 4) and ray parenchyma area (Fig. 5).

Most previous studies aiming to explore the growth and carbon balance response of trees to different regimes of water availability focused on simulating different levels of short-term drought (Irvine et al. 1998, Jyske et al. 2010, Klein et al. 2011, 2014, Montwé et al. 2014). In our research, as in a few others (Dobbertin et al. 2010, Martin-Benito et al. 2017, Timofeeva et al. 2017, von Arx et al. 2017), we instead aimed to investigate the long-term irrigation effects on radial growth and carbon storage in wood, specifically targeting mature Aleppo pines growing in the field and on a xeric site under semi-arid conditions. All trees presented similar year-to-year growth variations (Fig.1) which parallel the precipitation pattern already reported for the region (Pacheco et al. 2016). This represents the typical response of Aleppo pine growing in water-limited forests (Pasho et al. 2012, Klein et al. 2014, Sarris et al. 2013, Gazol et al. 2017) and supports the evident reaction of the species, even under moderate irrigation, to changes in soil water availability or precipitation. In the case of the TI trees, their tree-ring growth was double those in the IR plot during the irrigation period. This difference is explained given that IR trees were exposed to indirect irrigation due to their position next to an irrigated area, while TI trees were directly irrigated. After the years of watering, the tree-ring width values of TI trees fell immediately and synchronized with those of the other plots for the rest of the period, tending to maintain higher values than the control trees but not significantly different in most of the years. This supports the idea of a short-term influence of irrigation on the growth performances of the species, without any significant carryover or legacy effect.

Wood-anatomical traits provide further insight into the long-term effects of irrigation on tree functioning (Fig. 2). TI trees depicted a legacy effect of the irrigation treatment on lumen area, with 4 to 5 years of consistently higher values before returning to follow the same pattern as control trees. This reduction in lumen area is in line with the expected plastic response of xylem anatomy (tracheid expansion) to increase resistance to cavitation when drought conditions arise (Hacke et al. 2001, Hacke and Sperry 2001). No significant inter-annual changes were observed in cell wall thickness over the analyzed period, confirming that this trait is less sensitive than lumen area to environmental variability in drought-prone regions (Pacheco et al. 2016, 2017). The decadal averaged ring profiles (Fig. 3) permitted a better assessment in tune with long-term changes of wood-anatomical traits. With irrigation during the 1980s, TI trees showed an increase in both lumen area and cell-wall thickness that presented values similar to IR trees. In contrast to the tree-ring width data, the average ring profiles highlighted how in the two decades following the irrigation treatment, lumen areas of TI trees remained significantly larger than those of control trees. In the case of cell-wall thickness during the post-irrigation decades, TI trees maintained similar values to IR trees, but just along the first six sectors of the profile that matches the earlywood. Considering the tree characteristics shown in table 1 (similar age and height in IR and TI trees), allometric balance is likely to be the main reason for their similar wood-anatomical traits after the irrigation treatment. Having similar height and therefore similar root-to-leaves path length for water transport, trees will tend to produce cells with similar lumen area in order to optimize their hydraulic architecture and minimize resistance to water flow (Carrer et al. 2015). Nonetheless, while both groups of trees feature similar heights they do have significantly different diameter, suggesting that secondary growth has been more affected by water limitation than primary growth.

Stem sapwood NSC showed no significant differences between the rings formed during or after irrigation in the TI trees, nor was there a difference from the control (Fig. 4). The only significant difference we found was with IR trees, where the starch component of total NSC had a smaller percentage than any other group (Fig.4). This result, where trees in the wettest condition (IR) had a lower NSC concentration, but also less xylem area occupied by ray parenchyma (Fig. 5) than non-irrigated and TI ones, conforms more with the idea of drought-induced growth reductions than with a decline of carbon uptake (Granda and Camarero 2017, Pellizzari et al. 2016). These findings depict the long-term extension of what has been described in Aleppo pine by other authors working on the effects of short-term (two years) drought conditions (Klein et al. 2014); i.e. higher NSC levels in slow-growing trees subjected to dry conditions and lower levels in fast-growing unstressed trees. NSC concentrations are generally higher in actively growing tissues such as leaves, fine roots and phloem (Gruber et al. 2013, Hoch et al. 2003, Pruyn et al. 2005), and this could explain why sapwood NSC concentrations are significantly lower in our fast-growing IR trees. These results seem to indicate a dilution effect on NSC when considering the total biomass of IR trees compared to the other treatments. From the total NSC, the starch component is the one accounting for the significantly lower concentrations measured, which reinforces the assumption that the highly active areas of the crown require these rapidly available assimilates as soluble sugars to sustain growth, or that these sugars are used as osmolytes to equilibrate intra- and extra-cellular solute concentrations (Oribe et al. 2003).

Parenchyma in the stem sapwood stores a large proportion (25–40%) of the overall NSC reserves in a tree, which makes it a more important NSC reservoir than the phloem or leaves (Jacquet et al. 2014). Measuring the total percentage of ray area per year (Fig. 5), we tried to elucidate if irrigation affected long-term carbon allocation in the wood and if this anatomical variable could be considered a proxy for sapwood NSC concentrations. The lower percentage of ray parenchyma area in IR trees suggests that irrigation enhanced growth and reduced NSC concentration, however the increase in ray parenchyma was not proportional to the increase in xylem area occupied by tracheids. This result may indicate that higher water availability improves growth and tracheid expansion rather than the formation of ray parenchyma and the corresponding increase in NSC reserves. In addition, the TI trees had lower ray area percentages during the irrigation decade (1980s) and these percentages rose afterwards to match those of the control, which is in line with other researches on Scots pine (von Arx et al.

2017). During the studied period, the three wettest springs (1988, 2002 and 2008) confirm the key role of water availability not just on ring width, but also on parenchyma ray area, as shown in Fig. 5. This convergence of all treatments in those three years coincides with the peak growth rate for Aleppo pine in this location, as observed by Camarero et al. (2010) and Pacheco et al. (2017). Further research focusing on NSC pools could investigate whether the increase of NSC concentrations in non-irrigated trees compensates for their smaller diameter as compared with IR trees. We speculate that IR trees should have the highest NSC sapwood pools, but more detailed biomass data would be required to test this.

Growth, wood-anatomical traits and parenchyma measurements, but not NSC concentrations, showed significant differences between the irrigation and post-irrigation phases in TI trees. As NSC concentrations are not evenly distributed in the sapwood, but typically show a decrease from the outer to inner sapwood (Hoch et al. 2003, Saranpää and Höll 1989), this could explain why no significant differences were found in this case. However, it is also possible that when TI trees were first exposed to irrigation it allowed them to enhance photosynthesis and allocate relatively more NSC to growth than carbon storage, while still increasing their NSC pool size (von Arx et al. 2017). This could be considered as a strategy to increase NSC storage during good periods to withstand future droughts. Nevertheless, an open question is to discern if NSC pools changed as a function of irrigation, in parallel with the long-term changes to ray parenchyma suggesting an active long-term response. The decrease of NSC concentration in IR trees could in fact also be viewed as a passive "dilution effect".

4.6 Conclusions

Our 40-year record of tree-ring growth and wood anatomical traits has improved the general understanding of long-term responses to different water availability in the drought-tolerant Aleppo pine. We showed that xylem anatomy was affected by changes in the irrigation regime; that anatomical traits are able to promptly adapt their structure to cope with improved water availability but also that they can maintain a legacy effect. Irrigation enhanced radial growth, favored the formation of tracheids with wider lumen areas and thicker cell walls, reduced sapwood NSC concentration and the xylem area occupied by ray parenchyma. Our findings indicate that under changing environmental conditions drought-adapted tree species are able to efficiently coordinate the long-term balance between carbon storage and use (growth).

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SUPPLEMENTARY INFORMATION

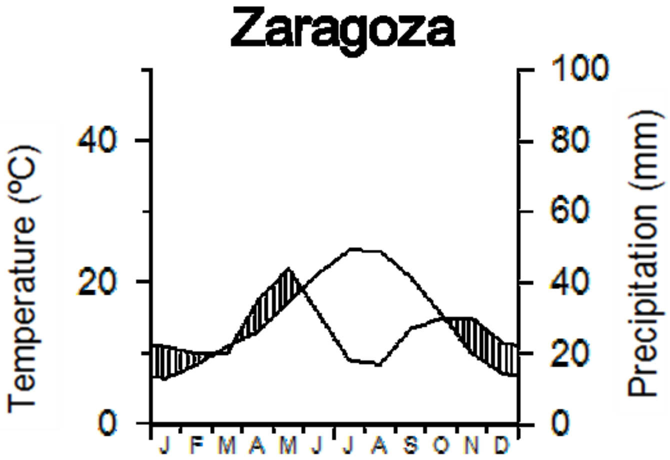

Figure S 4.1 Climate diagrams from Zaragoza's airport weather station showing data for the period 1950-2014.

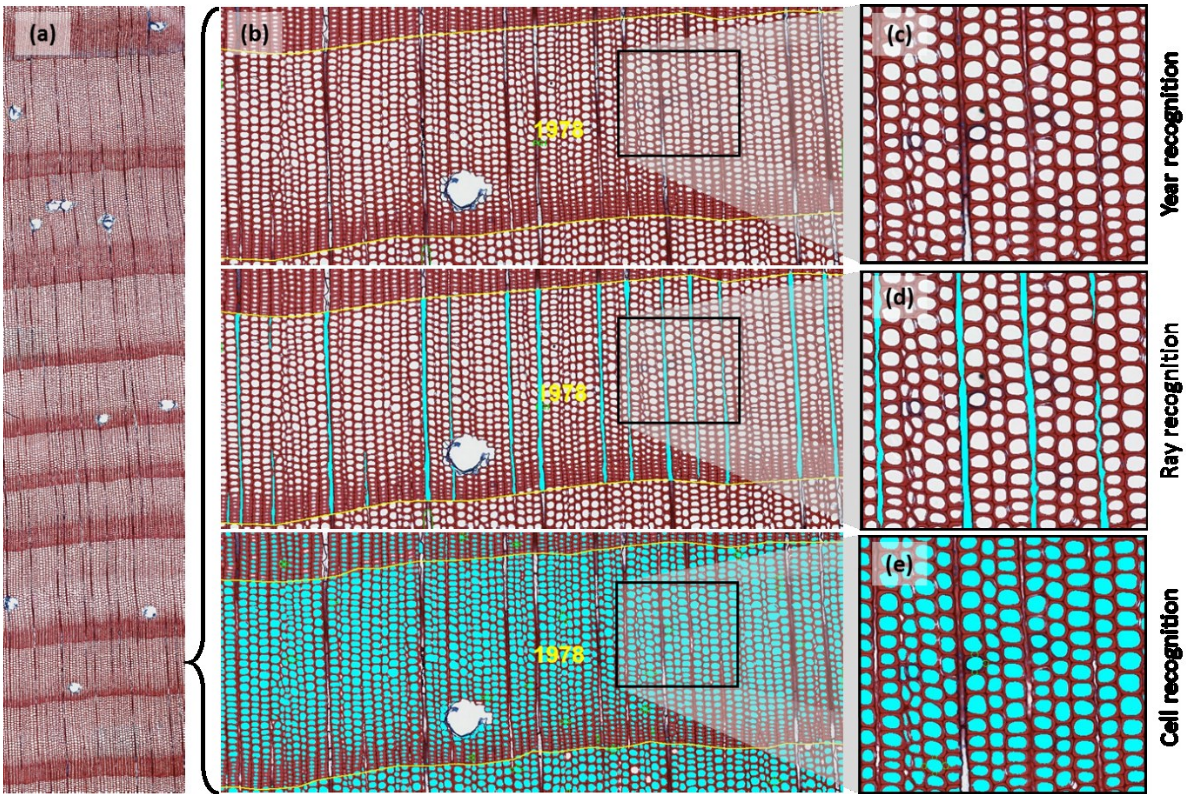

Figure S 4.2
Scheme of the semiautomatic image analysis performed by Roxas. (a) Panoramic image of tree core transversal section already coloured and fixed on a glass slide, (b) close-up of a single year three steps of anatomical recognition: (c) zoom on year recognition, (d) zoom on manual ray recognition and (e) zoom on automatic cell recognition**.**

Period	Treatment	Sectors (earlywood → latewood)									
		S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
		Lumen area (µm²)									
Irrigation	TI	742 ± 36.6	866 ± 41.6	916 ± 41.1	893 ± 42.8	821 ± 40.2	710 ± 37.8	605 ± 30.2	525 ± 27.7	418 ± 22.2	282 ± 14.4
	Control	434 ± 20.5	513 ± 26.8	547 ± 32.7	541 ± 33.5	512 ± 31.1	471 ± 27.8	388 ± 24.2	292 ± 20.8	206 ± 14.6	146 ± 7.8
	IR	797 ± 28.6	905 ± 29.7	935 ± 33.5	895 ± 32.1	784 ± 29.4	652 ± 29	527 ± 23.7	385 ± 19.6	282 ± 15.3	212 ± 10.5
Post-irrigation	TI	662 ± 27.3	747 ± 29.9	799 ± 29.2	812 ± 28.8	759 ± 30.7	693 ± 32.6	580 ± 29.2	431 ± 22.8	310 ± 15.2	190 ± 5.4
	Control	490 ± 19.5	539 ± 20.4	573 ± 21.7	592 ± 23.1	571 ± 23.9	528 ± 24	448 ± 23.3	356 ± 19.9	241 ± 14.1	169 ± 7
	IR	1046 ± 19.9	1150 ± 20.3	1199 ± 18.2	1133 ± 19	960 ± 21.3	750 ± 19.3	593 ± 16	481 ± 12.4	399 ± 12.4	265 ± 8.6
		Cell-wall thickness (µm)									
Irrigation	TI	5.17 ± 0.14	5.29 ± 0.15	5.45 ± 0.14	5.41 ± 0.13	5.41 ± 0.13	5.61 ± 0.14	5.87 ± 0.15	6.33 ± 0.14	7.02 ± 0.12	7.1 ± 0.12
	Control	4.6 ± 0.08	4.41 ± 0.08	4.39 ± 0.07	4.39 ± 0.08	4.46 ± 0.08	4.61 ± 0.08	4.72 ± 0.08	4.89 ± 0.09	5.05 ± 0.09	4.99 ± 0.09
	IR	5.31 ± 0.12	5.26 ± 0.13	5.25 ± 0.12	5.2 ± 0.12	5.32 ± 0.13	5.6 ± 0.14	6.06 ± 0.15	6.55 ± 0.16	7.07 ± 0.14	6.95 ± 0.13
Post-irrigation	TI	5.21 ± 0.08	5.11 ± 0.08	5.18 ± 0.08	5.27 ± 0.08	5.41 ± 0.08	5.6 ± 0.09	5.73 ± 0.09	5.99 ± 0.1	6.23 ± 0.12	6.15 ± 0.12
	Control	4.58 ± 0.06	4.42 ± 0.06	4.45 ± 0.06	4.52 ± 0.06	4.55 ± 0.06	4.59 ± 0.06	4.69 ± 0.06	4.83 ± 0.07	5.02 ± 0.08	5.06 ± 0.09
	IR	5.21 ± 0.06	5.15 ± 0.07	5.08 ± 0.06	5.04 ± 0.06	5.2 ± 0.06	5.58 ± 0.08	6.17 ± 0.08	6.85 ± 0.07	7.36 ± 0.07	7.33 ± 0.06

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5. General Conclusion

Throughout this thesis project, I performed a series of experiments using wood anatomy analysis and assessing the association with climate of several wood anatomical traits. In the same period, thanks also to the contribution of several other researchers, it came out how the field of quantitative wood anatomy and dendroanatomy is developing at fast rate and is becoming one of the most promising tools to increase our understanding on the tree response to climate change on the Mediterranean region and beyond.

Along the first phase of this research project, I compared the growth response to climate of two co-existing conifers contributing with new insights on the strategies used by each species to compete on the harsh dry environment of continental Mediterranean climate. The climatic correlation performed during this part of the research showed that *Pinus halepensis* besides relaying on spring precipitations, as *Juniperus thurifera* also does, it presents a particular significant and positive response to winter rainfall. This suggested that while Aleppo Pine with its deeper root system can reach long-term accumulate soil moisture from the winter months, *J. thurifera* has to relay in short-term soil water availability considering its shallow root system. In a broader aspect, it was reported that the relationships found between climate, growth and wood-anatomical data concur with the bimodal pattern of xylogenesis described for conifers in continental Mediterranean climates. This pattern is driven by the formation of latewood IADFs favoured by late-summer to early-autumn wet conditions which triggered the formation of tracheids with ample lumen areas. This specific trait represents a valuable ecological proxy of water availability during the lignification and maturation of tracheids in both species. After comparing the frequency on the formation of IADFs between the study species, Aleppo pine showed higher values suggesting once again that its accessibility to deeper soil water reserves allows it to maintain cell activity long enough to be able to take advantage of late summer or autumn rainy conditions. These interesting results and at the same time the lack of knowledge on the mechanics of the formation of the IADFs draw down the path to follow on the next phase of the research.

During the second part of my study I focus just in one specie, *Pinus halepensis*, growing in two distinct sites, to explore the species growth plasticity under different conditions within the Mediterranean region. It was found that growth bimodality and IADF formation, two facets of the double period of radial growth and wood formation in Mediterranean forests, were more pronounced in the mild-wet site (Barcelona) than in the cold-dry site (Zaragoza), which also displayed a shorter growing season. In the drier site, tracheid lumen area responded to short-term (weekly scales) changes in precipitation and soil water availability during the period when growth rates peaked, i.e. late spring to early summer. Furthermore, trees in the wetter Barcelona site formed wider lumen areas, which in addition to the longer growing season, could explain why they also presented higher growth rates, more IADFs and a more pronounced bimodal behaviour. All this results come in line with the previous suppositions made during the first part of the whole research. It can be said that milder conditions allowed us to observe then plasticity to adapt and take advantages of the changing conditions of Mediterranean weather.

Another important contribution of this part of the research was demonstrating that matching short-term intensive monitoring with long-term retrospective wood anatomy records can be an efficient combination for a quantitative and robust assessment of cambial phenology, IADFs and to define the climate drivers triggering the bimodal growth pattern in Mediterranean environment.

Lastly, on the third part of the study, we evaluate the long-term response of Aleppo pine to irrigation by including the quantification of non-structural carbohydrate concentration and the measurement of parenchyma area, to get a physiological insight on the tree growth behaviour along several decades.

This unique long-term manipulation trial permitted to objectively highlight the causal relationships between tree growth and the carbon supply at stem level and water availability. Results shown that trees tend to maintain a fairly good coordination between C supply and demand, and even during prolonged drought trees maintain a positive carbon balance. .In conclusion, this research has added important contributions to the general understanding of how the climatic conditions drive the dynamics of secondary growth in the intra-annual scale and has demonstrated the valuable input that wood anatomy analysis give in terms of validating these observations and finally permitting a higher time resolution of the inferences compared to the classical tree-ring approach but still with a retrospective long-term perspective.

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Research paper

Linking wood anatomy and xylogenesis allows pinpointing of climate and drought influences on growth of coexisting conifers in continental Mediterranean climate

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Forecasted warmer and drier conditions will probably lead to reduced growth rates and decreased carbon fixation in long-term woody pools in drought-prone areas. We therefore need a better understanding of how climate stressors such as drought constrain wood formation and drive changes in wood anatomy. Drying trends could lead to reduced growth if they are more intense in spring, when radial growth rates of conifers in continental Mediterranean climates peak. Since tree species from the aforementioned areas have to endure dry summers and also cold winters, we chose two coexisting species: Aleppo pine (*Pinus halepensis* Mill., Pinaceae) and Spanish juniper (*Juniperus thurifera* L., Cupressaceae) (10 randomly selected trees per species), to analyze how growth (tree-ring width) and wood-anatomical traits (lumen transversal area, cell-wall thickness, presence of intra-annual density fluctuations—IADFs—in the latewood) responded to climatic variables (minimum and maximum temperatures, precipitation, soil moisture deficit) calculated for different time intervals. Tree-ring width and mean lumen area showed similar year-to-year variability, which indicates that they encoded similar climatic signals. Wet and cool late-winter to early-spring conditions increased lumen area expansion, particularly in pine. In juniper, cell-wall thickness increased when early summer conditions became drier and the frequency of latewood IADFs increased in parallel with late-summer to early-autumn wet conditions. Thus, latewood IADFs of the juniper capture increased water availability during the late growing season, which is reflected in larger tracheid lumens. Soil water availability was one of the main drivers of wood formation and radial growth for the two species. These analyses allow long-term (several decades) growth and wood-anatomical responses to climate to be inferred at intra-annual scales, which agree with the growing patterns already described by xylogenesis approaches for the same species. A plastic bimodal growth behavior, driven by dry summer conditions, is coherent with the presented wood-anatomical data. The different wood-anatomical responses to drought stress are observed as IADFs with contrasting characteristics and responses to climate. These different responses suggest distinct capacities to access soil water between the two conifer species.

Keywords: intra-annual density fluctuation, *Juniperus thurifera*, *Pinus halepensis*, quantitative wood anatomy, radial growth, xylem.

Introduction

Anthropogenic climate warming represents a global challenge for forest productivity in drought-prone areas (Reichstein et al. 2013). Models predict a worldwide increase of mean air temperature of ~2–4 °C with significant drying over regions such as the Mediterranean basin (IPCC 2013). Climate warming is expected to magnify drought stress in that area by raising air temperatures and evapotranspiration rates, thus increasing the frequency and severity of droughts and heat waves (Meehl and Tebaldi 2004). Arid and hot conditions could be more intense during spring, when radial growth rates of most Mediterranean conifers peak (Camarero et al. 2010, Vicente-Serrano et al. 2010), with negative consequences on forest growth (Sarris et al. 2007) and related ecosystem services such as carbon fixation in long-lasting wood pools. In addition to summer drought, low winter temperatures also constrain forest growth in continental Mediterranean areas (Gimeno et al. 2012, Granda et al. 2014). Several conifer species respond to these dual stressors with a bimodal radial growth pattern, i.e., with higher wood-formation rates in periods with optimal climatic conditions, namely spring and autumn (Camarero et al. 2010). This double seasonal climatic stress also implies that spring and autumn climatic conditions can have contrasting effects (formation of earlywood-tracheids at the final part of the ring) on growth and wood-anatomical traits (e.g., tracheid lumen area (LA) and cell-wall thickness (CWT)) (De Luis et al. 2007, Olano et al. 2012, Pasho et al. 2012). In fact, the formation of intra-annual density fluctuations (IADFs) in Mediterranean conifers could be related to this bimodal pattern of growth. For instance, latewood IADFs are produced when climate induces the formation of earlywood-like tracheids with wide LAs and thin cell walls in summer or autumn when typically thick-wall tracheids with narrow lumen are formed (Campelo et al. 2007). Despite IADFs having been climatically characterized in many Mediterranean conifer species (Vieira et al. 2010, Novak et al. 2013), we still lack a more mechanistic approach to determining how their formation and anatomy respond to climate. A deeper understanding of the processes forming the IADFs justified them as a valuable wood-anatomical feature to be studied to link climate, xylogenesis and wood anatomy as advocated in previous research (Vaganov et al. 2006). Since the bimodal growth pattern has been described following cambium phenology over the course of a few years (cf. Camarero et al. 2010), longer records of IADF production and wood anatomy could identify whether that pattern is reflected in the responses of xylem traits to climate variability. Specifically, it could be investigated whether LA or CWT respond differently to seasonally varying climatic stress.

The long-term climate effects on radial growth of conifers are usually analyzed by measuring annually or seasonally resolved variables such as tree-ring, earlywood or latewood widths using dendrochronology (Fritts 2001). However, quantitative wood anatomy allows information to be obtained on tree functioning (e.g., carbon uptake, water use) and growth to be encoded at much finer temporal scales (Fonti et al. 2010, Von Arx and Carrer 2014). Here, we aim to characterize how climate influences growth and wood anatomy in two coexisting conifer species (Spanish juniper and Aleppo pine) subjected to continental Mediterranean conditions but presenting different phenological (e.g., xylogenesis), morphological (e.g., root depth) and physiological (e.g., drought tolerance) features (Gauquelin et al. 1999, Ne'eman and Trabaud 2000, Baquedano and Castillo 2007, Camarero et al. 2010, DeSoto et al. 2011). To achieve this, we quantify wood-anatomical traits (LA, CWT) and relate them to climate variables (temperature, precipitation, water balance, vapor pressure deficit (VPD), soil moisture) by contrasting correlation analyses results and xylogenesis data available for both species. We also analyze how climate drives seasonal changes in wood anatomy by evaluating the formation of IADFs in response to specific climatic conditions. We hypothesize that the bimodal pattern of growth is idiosyncratically expressed by the two coexisting conifer species, which should show different growth and wood-anatomy responses to climate. We discuss how these different wood responses to climate relate to contrasting species-specific traits, mainly those related to the root depth and the ability of trees to access soil water.

Materials and methods

Study site and species

We selected a mixed Mediterranean forest (Vedado de Peñaflor) located close to Zaragoza city (41°47′N, 0°43′W, 560 m above sea level (a.s.l.)), in the Middle Ebro Basin, northeastern Spain. Based on climate data for the period 1970–2013 (see below), the climate at the site is continental Mediterranean with an average yearly rainfall of 313 mm (semi-arid conditions), of which 66 mm were recorded between June and August. Annual mean temperature was 13.8 °C with frosts occurring frequently during winter (see Online Resource 1, Appendix S1 available as Supplementary Data at *Tree Physiology* Online). The estimated annual water balance was −476 mm with water deficit occurring from March to October. No significant trend was observed in precipitation data of the study area since 1970, but mean maximum temperatures have been steadily rising leading to drier conditions (see Appendix S1 available as Supplementary Data at *Tree Physiology* Online). Gypsum and marls comprise the parent rock material, while the topography of the terrain consists of small hills and valleys with west and east-facing slopes. Two co-occurring evergreen conifer species belonging to different families were selected: Spanish juniper (*Juniperus thurifera* L., Cupressaceae) and Aleppo pine (*Pinus halepensis* Mill., Pinaceae). Spanish juniper is endemic to the Western Mediterranean basin, being most abundant in Spain and Morocco, and it usually grows under continental Mediterranean climate conditions on poor or rocky soils (Gauquelin et al. 1999). Spanish juniper growth is enhanced by wet winter and warm spring conditions, whereas summer water deficit is associated with low growth rates (DeSoto et al. 2012). Aleppo pine is the most widely distributed Mediterranean pine species and is well adapted to growth under xeric conditions by tolerating water shortage (Ne'eman and Trabaud 2000). In the study area, Aleppo pine growth is mainly enhanced by wet and cool conditions during spring and in the winter prior to tree-ring formation (Pasho et al. 2012). At the studied site, *J. thurifera* forms a relict population mostly located on valley bottoms and west-facing slopes, and is intermingled with *P. halepensis*, which is mostly present on east-facing slopes where xeric conditions are stronger. More details on the study site can be found in Camarero et al. (2010).

Climate data

Daily climatic records were obtained from the Peñaflor-Aula Dei (41°45′N, 0°49′W, 280 m a.s.l.) weather station located at 5 km from the study site. In order to fill some missing temperature data gaps from Peñaflor we used the Zaragoza-airport (41°40′N, 1°00′W, 263 m a.s.l.) weather station located at 30 km from the study site. We considered the daily-resolved records for mean, maximum and minimum temperatures, relative humidity and total precipitation for the period 1970–2013. Vapor pressure deficit was estimated using temperature and humidity data (Abtew and Melesse 2013). We also calculated the water balance or difference between precipitation and potential evapotranspiration (PET) following Hargreaves and Samani (1982), and the soil moisture deficit (SMD) was obtained as the difference between cumulative evapotranspiration and cumulative rainfall (Cocozza et al. 2012). Daily data were also averaged (temperatures, VPD) or summed (precipitation, water balance) at various time intervals (5, 7, 10 and 15 days) to analyze climate–growth relationships at multiple temporal scales.

Dendrometer and xylogenesis data

To characterize the phenology of radial growth and xylogenesis (phases of xylem formation) we used data of radial stem changes based on manual band dendrometer records (Agricultural Electronics, Tucson, AZ, USA) and observations of xylem formation (xylogenesis) based on repeated wood sampling (micro cores), respectively. These data were recorded biweekly or monthly in 10 trees per species. Part of the dendrometer data (2006–12 period) and all xylogenesis data (years 2006 and 2010) have been already published (Camarero et al. 2010, Pasho et al. 2012). Dendrometer data were used to determine the times of maximum increment and decrement rates that were defined when >50% of trees showed positive or negative increment rates, respectively. Xylogenesis allowed the main phases of xylem formation to be characterized in both species by counting different cell developmental phases (cambium cells, radially enlarging tracheids indicating cambium resumption, thickening tracheids indicating the onset of wall lignification, and mature tracheids (cf. Antonova and Stasova 1993, Cuny et al. 2014). More details on these data can be found in Camarero et al. (2010) and Pasho et al. (2012).

Wood-anatomy data acquisition and analyses

In January 2014, a total of 20 dominant trees, between 40 and 60 years old (10 per species), were randomly selected and sampled with a Pressler increment borer taking three radial cores per tree at 1–1.3 m. During sampling we avoided damaged stem areas with visible scars or showing signs of recent injuries and forming compression wood. In the laboratory, two cores per tree were prepared following standard dendrochronological methods (fixed in woody mounts, glued and sanded). These samples were visually cross-dated and then measured to a precision of 0.01 mm using a LINTAB measuring system (F. Rinn, Heidelberg, Germany). Cross-dating was statistically validated using the COFECHA program (Holmes 1983). For histological analyses, we selected the five best cross-dated trees cores per species, that is, trees with cores showing the highest correlation with the mean ring-width series of each tree species. We also considered cores without visible anomalies (e.g., reaction wood, resin pockets) and containing the pith in order to obtain complete tree-ring sequences. Samples were processed following Gärtner and Schweingruber (2013). Cores were cut in to small pieces (3–5 cm long), boiled in water to soften the wood and then cut in to thin slices (10–20 μm thick) using a rotary microtome. Slices were then cleaned, stained with a safranin (1%) and astra blue (0.5%) (both in distilled water), rinsed with water and alcohol and finally permanently fixed with a synthetic resin (Eukitt™; Merck, Darmstadt, Germany).

Image acquisition was performed with a digital camera mounted on a light microscope; images were captured at 100× magnification. Overlapping images were taken from each sample and stitched together using the PTGui software (New House Internet Services, Rotterdam, The Netherlands) to obtain high-resolution images (0.833 pixels μm^{-1}) of the entire section. Tree-ring borders were manually drawn on the images that were semi-automatically analyzed using the ROXAS software (Von Arx and Carrer 2014).

We measured the following wood-anatomical traits in the transversal xylem sections: mean LA and radial CWT. The radial dimension of CWT was chosen because it has a higher year-to-year variability than the tangential dimension (Vysotskaya and Vaganov 1989). Then, following Hereş et al. (2014), we calculated several parameters related to the hydraulic performance of the tree: (i) mean hydraulic diameter (d_h); (ii) mean percentage conductive area; and (iii) theoretical hydraulic conductivity (K_h) computed according to the Hagen–Poiseuille law (Tyree and Zimmermann 2002). The d_h was calculated by assuming that LAs are circular and using the formula $d_h = \Sigma d^5/\Sigma d^4$, where d is the tracheid diameter (Pockman and Sperry 1997).

To better define the time windows over the phases of radial growth we created 10 chronologies out of LA and CWT based on the relative radial position of each tracheid along the tree ring. This was done by dividing each tree ring into 10 sectors (deciles) along the radial direction and then computing the median LA and CWT values for each decile (see Online Resource 2, Figure S2 available as Supplementary Data at *Tree Physiology* Online). We decided to use deciles because they provided a good trade-off between sample size (number of cells per sector) and spatial and temporal resolution.

To assess the presence of latewood IADFs and calculate their relative frequency (%), we graphed the LA and CWT mean values along the tree-ring for each IADF and compared them with the Mork index, which is commonly used to define the latewood presence (Denne 1988). In this case, the Mork index proved to have a low efficiency in accurately defining earlywood and latewood limits due to the presence of latewood IADFs (see Online Resource 3, Figure S3 available as Supplementary Data at *Tree Physiology* Online). Intra-annual density fluctuations exclusively found within the latewood were therefore identified visually.

Lastly, we built chronologies of tree-ring width and wood-anatomical variables for each tree species using the ARSTAN program, which was specifically developed for the removal of biologically induced age–size related growth trends (Cook 1985), tendencies that are also present in wood-anatomical data (Carrer et al. 2015). Individual series were fitted using a cubic smoothing splines function with 50% frequency–response cutoff of 50 years. Afterwards, observed values were divided by expected values to obtain detrended and dimensionless indices, which were then subjected to autoregressive modelling to remove part of the temporal autocorrelation. These indices were averaged to create the mean chronology of each variable for the two species.

The climate–growth associations were quantified by calculating Pearson correlations between the detrended growth and wood-anatomical chronologies and the different time windows of climatic data for the best-replicated period (1975–2013). In the case of IADF frequencies, we related them to climatic variables using the Spearman non-parametric correlation coefficient (r_s). The calculations were computed from previous October to current September based on a previous study (Camarero et al. 2010).

Results

Wood-anatomical features

The quantitative description of wood anatomy was based on rings formed between 1970 and 2013 and included the measurement of 3.0×10^5 and 4.6×10^5 tracheids in Aleppo pine and Spanish juniper, respectively. The two species significantly differ in the distributions of LA and CWT (Figure 1), with pine typically forming tracheids with larger lumens (pine 276 μm^2 vs juniper 147 μm^2) and thicker walls (pine 4.1 μm vs juniper 3.4 μm) (Table 1). Consequently, both the d_h and K_h were higher in Aleppo pine than in Spanish juniper, despite the fact that mean conductive area (34%) did not significantly differ between the two species (Table 1). The chronologies based on these anatomical variables showed lower correlation values between conspecific trees as compared with tree-ring width series, except for K_h. The annual mean production of IADFs in Spanish juniper was twice (32%) that in Aleppo pine (15%), but the mean IADF series of both species were highly related ($r = 0.33$, $P = 0.04$) indicating that they encoded similar climatic information.

We found a highly significant ($P < 0.001$) relationship between tree-ring width and the number of tracheids forming a ring, slightly

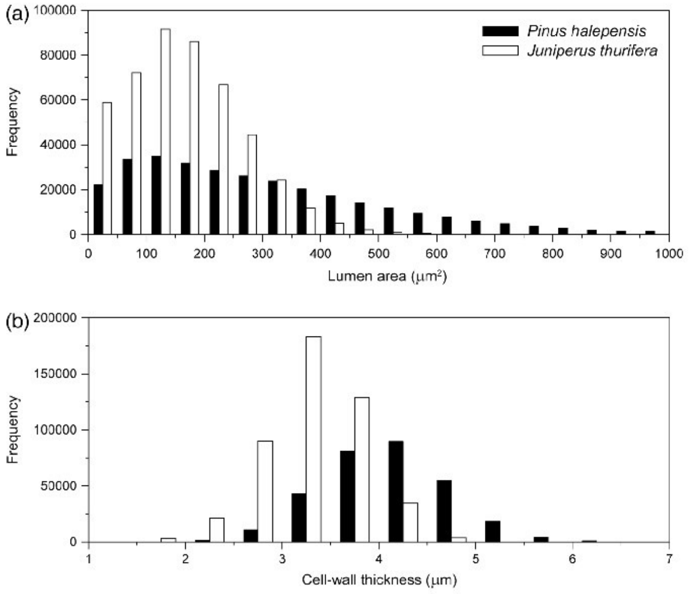

Figure 1. Distribution of (a) tracheid LA and (b) CWT for the two conifers (Aleppo pine, *P. halepensis*; Spanish juniper, *J. thurifera*). Data correspond to the 1975–2013 period.

Table 1. Main descriptive variables obtained for the trees considering size, age, tree-ring width and wood-anatomical variables. Values are means and standard deviation calculated for the period 1970–2013. Different letters indicate significant ($P < 0.05$) differences between species based on Mann–Whitney *U*-tests. IADF, intra-annual density fluctuation; LA, lumen area; CWT, cell-wall thickness; d_h, hydraulic diameter; K_h, theoretical hydraulic conductivity.

Variables	Spanish juniper (*J. thurifera*)		Aleppo pine (*P. halepensis*)	
	Variable	Correlation between trees	Variable	Correlation between trees
Diameter at 1.3 cm (cm)	14.8 ± 1.0	–	16.7 ± 1.0	–
Tree height (m)	5.1 ± 0.2a	–	6.0 ± 0.2b	–
Age at 1.3 m (years)	60 ± 15	–	54 ± 12	–
Tree-ring width (mm)	1.26 ± 0.72	0.42	1.82 ± 1.14	0.53
Frequency of IADFs (% year^{-1})	32 ± 20b		15 ± 12a	
No. tracheids per ring	4264 ± 1053		2966 ± 835	
LA (μm^2)	147.26 ± 36.55a	0.14	275.78 ± 86.46b	0.22
CWT (μm)	3.43 ± 0.31a	0.28	4.14 ± 0.36b	0.10
d_h (μm)	17.00 ± 1.97a	0.30	24.82 ± 4.48b	0.39
K_h (kg m MPa^{-1} s^{-1} 10^{-13})	6.22 ± 3.93a	0.45	12.50 ± 7.59b	0.60
Conductive area (%)	33.58 ± 6.34	0.24	34.44 ± 5.47	0.25

stronger in juniper ($r = 0.93$, $n = 244$) than in pine ($r = 0.90$, $n = 179$). As expected, for a similar number of tracheids pine formed rings approximately twice the width of those of juniper (see Online Resource 4, Figure S4 available as Supplementary Data at *Tree Physiology* Online). In addition, d_h and CWT were negatively related in juniper ($r = -0.40$, $n = 237$, $P = 0.015$), whereas this association was positive in pine ($r = 0.26$, $n = 176$, $P < 0.001$).

Phenology of radial growth and xylem formation

On average, both species started growing in early April and finished in late October (Table 2). Maximum radial growth increment rates were observed in mid (Spanish juniper) to late May (Aleppo pine), whilst maximum stem decrement rates were detected in early (Spanish juniper) to late August (Aleppo pine) in response to summer drought. Xylogenesis data showed that the first tracheids were formed in late March in both species, i.e., almost 15 days before the first stem radial increment was detected (Table 2). However, the maximum xylem growth rates, corresponding to the formation of radially enlarging tracheids, occurred in mid-to late May, and they almost coincided with those observed using dendrometers. The wall-thickening tracheids started and finished forming from early May to mid-October in Aleppo pine, and from late May to late October in Spanish juniper.

Inter-annual temporal patterns in growth and wood anatomy

Raw chronologies of tree-ring width and LA showed negative and positive significant ($P < 0.001$) trends, respectively, in both species (pine, $r = -0.45$ and $r = 0.77$; juniper, $r = -0.62$ and $r = 0.46$). The year-to-year variability in ring width was similar between the two species ($r = 0.73$, $P < 0.001$) indicating a likely common climatic signal (Figure 2). In fact, growth peaked in years characterized by wet spring conditions (e.g., 1997) whilst wood production was very low during years with a warm and dry spring (e.g., 2005; see Online Resource 1 available as Supplementary Data at *Tree Physiology* Online). The patterns of mean LA, per ring, were also consistent over time between the two coexisting species ($r = 0.71$, $P < 0.001$), but this was not the case for CWT ($r = 0.23$, $P < 0.001$).

Climate influences on wood anatomy

The results obtained using a 15-day window produced the most robust and significant correlations. In general wet and cool conditions during the previous winter (recharge of soil water) and in spring (Spanish juniper) or summer (Aleppo pine) enhanced LA expansion, while dry spring-to-summer conditions (negative water balance) reduced the conduit size (see Online Resource 5, Figure S5 available as Supplementary Data at *Tree Physiology* Online). Warm summer to autumn conditions preceded by low SMD enhanced CWT in the case of Spanish juniper, whereas wet and warm late-winter and summer conditions were associated with high CWT values in Aleppo pine. The first of these two associations was also observed in juniper. Considering the analyses based on tree-ring deciles, wet conditions from February to July enhanced LA of tracheids located in the first four deciles (early to mid-earlywood) and also in the last decile (late latewood) in both species, with maximum positive associations observed in May and June (Figure 3).

Contrastingly, the positive role of warm February conditions on LA affected most deciles distributed throughout the ring in juniper, but mainly the fifth to sixth deciles in the pine (mid- to late earlywood). In the case of CWT, warm February minimum temperatures enhanced wall thickening in Aleppo pine along the entire ring but the signal was stronger in the first three and last two deciles. Wet February to March conditions also enhanced CWT in both species, but July to September wet conditions only increased CWT in the last two deciles (latewood) of pine tracheids.

Table 2. Dates (day of the year) summarizing the xylem phenology (xylogenesis) of the two studied species (Spanish juniper, *J. thurifera*; Aleppo pine, *P. halepensis*) in the study site based on manual band dendrometers (2006–12 period) or xylogenesis (2006 and 2010) data. Abbreviations referring to dendrometer data: F, first positive growth rate, MG, maximum radial increment rate; MD, maximum radial decrement rate; L, last positive rate. Abbreviations of xylogenesis phases: EE, start of growth (first enlarging tracheids are formed); ME, maximum production of radially enlarging tracheids; EL, beginning of wall-thickening tracheids; LL, last wall-thickening tracheids are formed.

Year	Dendrometer data								Xylogenesis data							
	J. thurifera				*P. halepensis*				*J. thurifera*				*P. halepensis*			
	F	MG	MD	L	F	MG	MD	L	EE	ME	EL	LL	EE	ME	EL	LL
2006	112	163	201	334	110	175	195	304	95	133	142	315	75	155	135	290
2007	115	141	200	292	90	134	257	290								
2008	102	136	247	302	103	152	255	307								
2009	90	135	205	272	89	135	225	330								
2010	97	131	183	321	114	160	257	322	88	137	150	290	88	142	122	285
2011	94	124	257	331	90	134	260	290								
2012	103	132	222	278	94	131	221	296								
Mean	102	137	216	304	99	146	239	306	92	135	146	303	82	149	129	288
SD	30	37	34	17	10	17	25	16	5	3	6	18	9	9	9	4
Julian day	11 April	16 May	3 August	30 October	7 April	24 May	25 August	31 October	31 March	14 May	25 May	28 October	21 March	27 May	7 May	13 October

Climatic drivers of IADF formation

A positive water balance in late July (pine) or early September (juniper) increased the formation of IADFs in the latewood (Figure 4). However, cold conditions in March to June and in autumn (only in juniper) were negatively related to IADF formation. Lastly, a higher SMD in autumn was associated to the production of IADFs, being more important from September to October in the case of juniper. Considering monthly values, in pine IADF frequency was positively related to wet July to August conditions, while in juniper IADFs were formed in response to years with wet (high water balance and low VPD) August conditions (Figure 5).

Discussion

Here we evidence that wood-anatomical features encode long-term and high-resolution climatic information on tree functioning and growth of two conifer species coexisting under continental Mediterranean conditions. The observed relationships between climate and anatomical traits confirm previous information on xylogenesis of the two species. This allowed the major wood responses to water shortage to be pinpointed. We found that wet conditions during the early growing season in spring or before (winter) result in the formation of wide tree rings that consist of numerous tracheids with relatively large lumens in the case of pine, while LAs are smaller in juniper. The small LAs of juniper species make them less vulnerable to cavitation as compared with coexisting pine species forming tracheids with larger lumens (Willson et al. 2008). This seems to explain why drought-induced die-off mainly affects pines in mixed pine–juniper forests on semi-arid sites (McDowell et al. 2008). However, not only tracheid dimensions but also small alterations on pit traits (frequency, geometry and biomechanics) between coexisting species can affect tree performance on water transport, embolism resistance and capacitance (Hacke 2015). Considering only our measurements, wood-anatomical data therefore show that junipers will be less vulnerable to die-off than pines since many Cupressaceae have evolved towards a drought-resistant xylem (Pittermann et al. 2012). On the contrary, pines will show higher growth rates and K_h if conditions are wet enough in the early growing season.

As it ages Aleppo pine forms wider tracheids compared with Spanish juniper. This is also related to the usual bigger size of adult trees (tree height and deeper root system (Willson et al. 2008)). The shallower root system of junipers could make them more dependent on summer and autumn rains than Aleppo pine, and this could explain why junipers tend to form latewood IADFs more frequently (Table 1) whereas pines are able to sustain low growth rates during the summer dry period (see also Camarero et al. 2010). This agrees with the different microsite location of the species on the study site. The

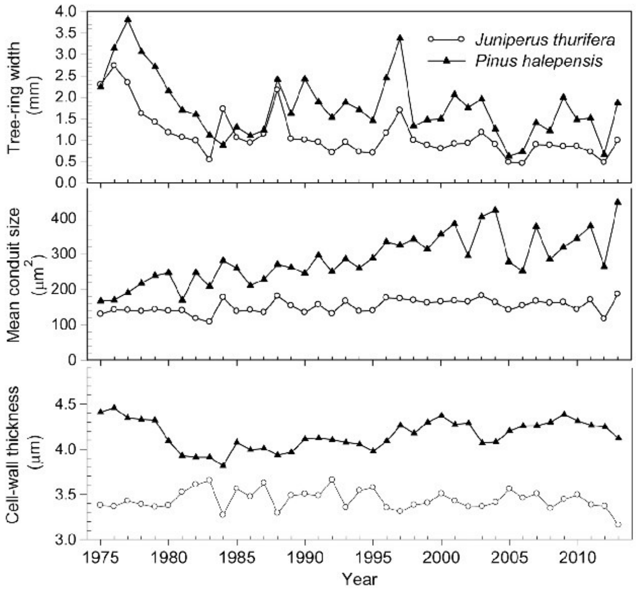

Figure 2. Mean tree-ring width, LA (mean conduit size) and CWT chronologies for the two species (Spanish juniper, *J. thurifera*; Aleppo pine, *P. halepensis*).

soil-water conditions play a central role from late March to June for the expansion of tracheid lumens in both species (Figure 3). However, the responsiveness of LA to SMD lasted until summer in juniper. In the case of CWT, correlations with climate were more variable than in the case of LA and differed between the two species. In both species, wet and warm late-winter conditions were associated with thicker earlywood and latewood tracheids, confirming lagged climate–anatomy relationships since latewood starts forming from the end of May onwards (Table 2). Warm summer to autumn conditions preceded by low soil moisture enhanced CWT in the case of Spanish juniper, whereas wet summer conditions were associated with thick cell walls in Aleppo pine. Such a response has been explained by the low intercellular variability in wall thickness among neighboring tracheids in Spanish juniper (Olano et al. 2012). These authors attributed the formation of latewood tracheids with smaller LAs in this species to limited carbohydrate availability because of competition with other carbon sinks (Oribe et al. 2003). However, our interpretation is that the amount of carbon invested for each tracheid is fairly similar over the entire growing season and the change in LA from earlywood to latewood is mainly responsible for the changes in CWT, which could represent an allometric relationship related to constraints of conduit size and wall reinforcement (Hacke et al. 2001), and not a carbon-related limitation of xylogenesis (Cuny et al. 2014). This argument is also supported for the pine species based on the associations observed between LA or hydraulic diameter and CWT (see Online Resource 4, Figure S4 available as Supplementary Data at *Tree Physiology* Online).

Soil water availability during the early growing season, which depends on precipitation but also on soil temperatures and water-holding capacity, is one of the main drivers of wood formation and radial growth under continental Mediterranean climates (Martin-Benito et al. 2013). We found that wet and cool May conditions at a 15-day scale enhanced lumen expansion in both species as a result of improved cumulative soil water reserves, but high precipitation in June was associated with narrow lumens, particularly in Aleppo pine (see Online Resource 5, Figure S5 available as Supplementary Data at *Tree Physiology* Online). At a monthly scale, Aleppo pine formed larger lumens in response to a high water balance during the early spring when xylem formation starts (Figure 3, Table 1), which is a similar signal to that observed for the tree-ring width (Pasho et al. 2012). This implies that those climatic conditions favorable to lumen enlargement also enhance radial growth and suggests that wood formation and a higher K_h in the early growing season are interrelated and largely determine the annual growth rate in this species. These findings confirm the importance of winter soil-water recharge for Aleppo pine prior to cambial resumption in spring and also suggest that this species can reach deeper soil-water pools than those accessible to junipers (Sarris et al. 2013). This vertical segregation of soil-water resources

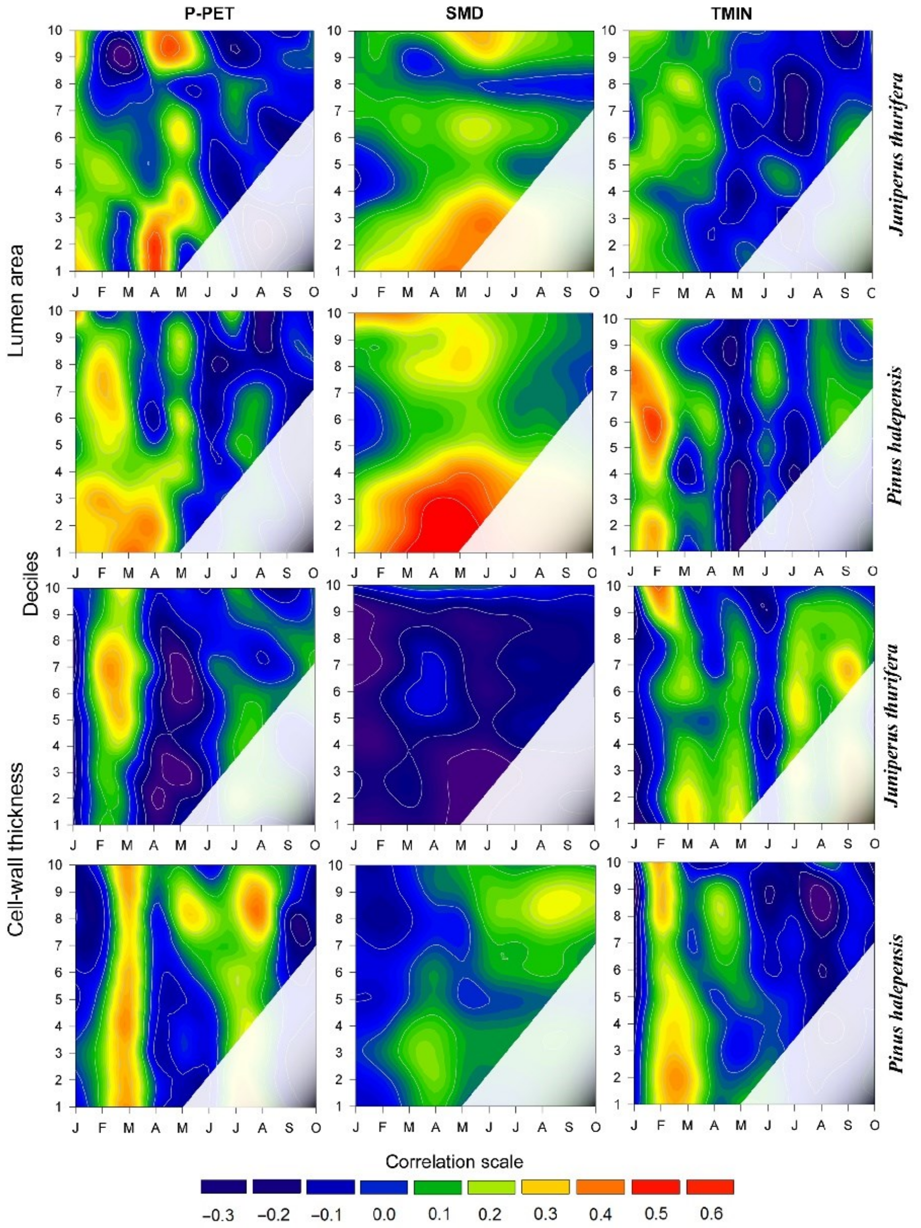

Figure 3. Climate–growth associations computed by comparing LA and CWT with monthly climatic variables (P-PET, water balance; SMD, soil moisture deficit; and T_{min}, minimum temperature) in Spanish juniper (*J. thurifera*) and Aleppo pine (*P. halepensis*). Correlations were calculated from January to October of the year of tree-ring formation (*x* axes) and partitioning the tree-ring in deciles along the radial direction from the beginning (Decile 1) to the end (Decile 10) of the ring (*y* axes). Correlations coefficient above $|0.31|$ are significant at $P < 0.05$. Chronologically inconsistent correlations (bottom-right triangle in each plot) are not shown.

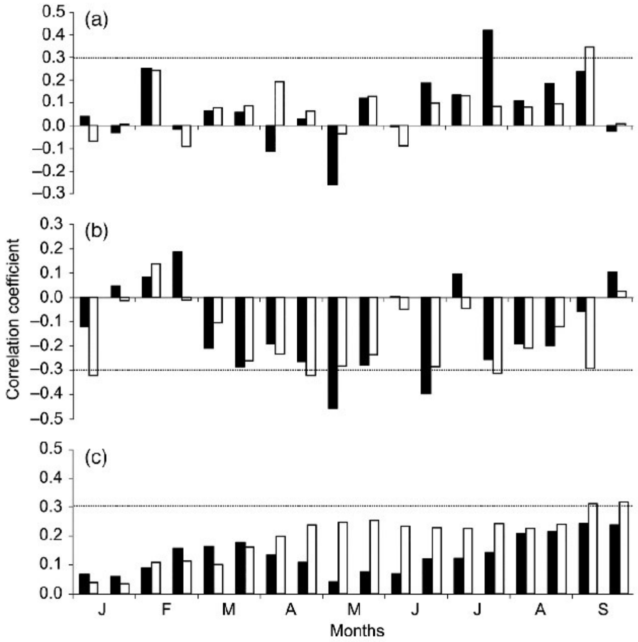

Figure 4. Spearman correlations computed for the two species (filled bars, Aleppo pine, *P. halepensis*; empty bars, Spanish juniper, *J. thurifera*) by relating the frequency of IADF and the 15-day summed water balance (a), averaged mean minimum temperatures (b) and summed SMD (c). Horizontal lines indicate significance levels at $P < 0.05$.

would explain the observed growth behavior differences by allowing a minimal growth of Aleppo pine in summer, while making juniper more dependent on spring rainfall to grow (Figure 3).

The longer season of latewood formation would explain a most active cambial activity of juniper in autumn and the frequent formation of IADFs. The positive effect of spring precipitation and summer SMD on the LA of juniper latewood tracheids indicates that the main driver of IADFs in this species is an increase in the water balance and a decrease on VPD, i.e., a reduction in soil and atmospheric dry conditions, which leads to the formation of tracheids with ample LAs in late summer and early autumn. This clearly represents a carryover effect in juniper response having first the punctual events of spring precipitations inducing a long-lasting effect on soil moisture which finally permits the species to produce the second peak of cambial activity. Contrastingly, latewood IADF formation in Aleppo pine is mainly favored by high precipitation in mid-summer in agreement with its earlier latewood development (Camarero et al. 2010).

The formation of latewood IADFs represents a valuable ecological proxy of late-summer to early-autumn water availability, although this is still little explored and IADF formation is not fully mechanistically understood. The relatively rapid formation of wide-lumen tracheids within the latewood suggests that the cambium, apparently dormant during the summer drought, reacts quickly to a significant amount of precipitation by forming an IADF. However, this response is similar to what is observed in the earlywood despite both occurring in completely different xylogenetic phases, namely radial enlargement of earlywood tracheids in the early growing season vs active lignification and maturation in the late growing season.

To conclude, tree-ring width and LA responded to wet previous winter to current spring conditions, respectively, in two conifers of different genera coexisting under continental Mediterranean conditions. The formation of latewood IADFs was favored by late-summer to early-autumn wet conditions which triggered the formation of tracheids with ample LAs. The relationships found between climate, growth and wood-anatomical data concur with the bimodal pattern of xylogenesis described for conifers in continental Mediterranean climates. The plastic responses of wood-anatomical variables to climate, and particularly to water deficit, could be caused by the different accessibility of soil water reserves for the species since junipers develop more shallow root systems than pines.

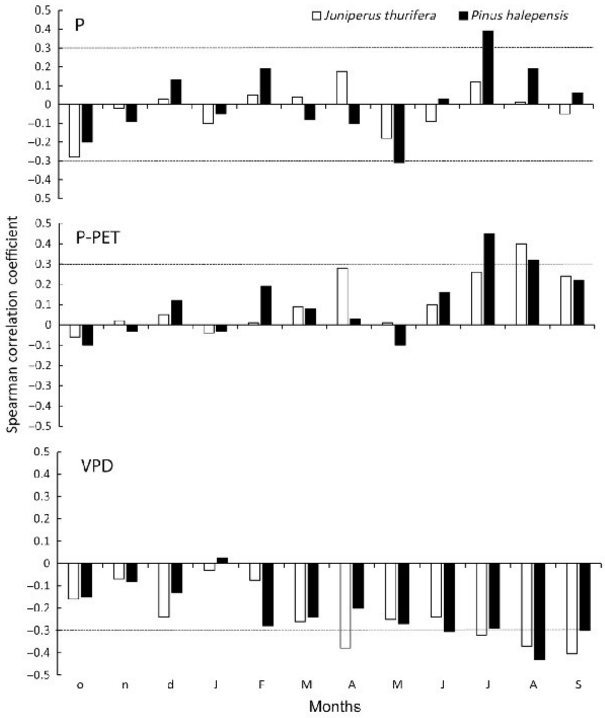

Figure 5. Relationships calculated between the frequency of latewood IADF of Aleppo pine (*P. halepensis*, filled bars) and Spanish juniper (*J. thurifera*, empty bars) vs monthly climatic variables: precipitation (P), water balance (P-PET) and VPD. Climatic variables include the time window from previous October to current September. Months prior to or concurrent with the growing season are abbreviated by lowercase and uppercase letters, respectively. Bars located outside the horizontal lines indicate significant correlations ($P < 0.05$).

Supplementary data

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

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Conflict of interest

None declared.

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Disentangling the climate-driven bimodal growth pattern in coastal and continental Mediterranean pine stands

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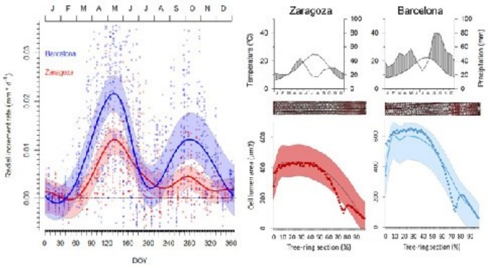
HIGHLIGHTS

- Dendrometers and dendroanatomy are used to compare Aleppo pine growth dynamics.
- Trees in the wetter coastal site show a stronger bimodal growth pattern.
- Intra-annual density fluctuations are less frequent in the drier inland site.
- Growth bimodality and intra-annual density fluctuations share common climatic cues.

GRAPHICAL ABSTRACT



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ABSTRACT

Mediterranean climate promotes two distinct growth peaks separated by summer quiescence in trees. This bimodal pattern has been associated to favourable growing conditions during spring and autumn when mild temperatures and soil-water availability enhance cambial activity. Climatic models predict progressive warming and drying for the Mediterranean Basin, which could shorten or shift the spring and autumn growing seasons. We explored this idea by comparing two sites with different Mediterranean climate types (continental/dry and coastal/wet) and studied how climate drives the bimodal growth pattern in Aleppo pine (*Pinus halepensis*). Specifically we investigated the intra-annual changes in wood anatomy and the corresponding formation of density fluctuations (IADF). Trees on both sites were analyzed by dendrometer monitoring and by developing chronologies of wood anatomical traits. Radial-increment dynamics followed a similar bimodal pattern in both sites but coastal trees showed higher increments during the spring and autumn growth peaks, especially in autumn. The summer rest of cambium activity occurs almost one month earlier in the coastal than in the inland site. Lumen area and cell-wall thickness were significantly smaller in the continental site, while the increment rate of cell-wall thickness during an IADF event was much higher in the coastal pines. The accumulated soil moisture deficit was the main climatic constraint of tracheid enlargement in continental pines. Intra-annual density fluctuations were more frequent in the coastal trees where wood anatomy features recover to average values after such events, meanwhile inland trees presented a much lower recovery rate. Growth bimodality and the formation of density fluctuations were linked, but mild climate of the coastal site allows a longer growing season, which explains why trees in this area showed higher and more variable growth rates.

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1. Introduction

In Mediterranean regions of the northern Hemisphere many tree species present, within the same growing season, two periods of growth activity, one in spring and a second in autumn. This bimodal pattern has been associated to favourable growing conditions, like mild temperatures and adequate soil-water availability (Mitrakos, 1980). In between these growth periods, low winter temperature and summer drought can limit or even pause cambium activity (Ribas, 2006; Camarero et al., 2010; Gutiérrez et al., 2011; Touchan et al., 2012; Prislan et al., 2016). This growth rest allows trees to acclimatize to summer droughts, which, although very variable in duration and intensity between regions and years, are typical of Mediterranean climates and greatly affect forest productivity (Girard et al., 2012).

This bimodal growth pattern can usually leave a permanent imprint in xylem with the formation of a distinctive anatomical wood features such as intra-annual density fluctuations (hereafter IADFs). These tree-ring features (Fritts, 2001; De Micco et al., 2014) represent a relevant wood trait to identify bimodality in Mediterranean tree species (Cherubini et al., 2003). IADFs are formed when limiting conditions interrupt normal radial growth, producing latewood-like cells (usually with narrow lumens and thick walls) within the earlywood or, more frequently, earlywood-like cells (usually with wide lumens and thin walls) within the latewood (Campelo et al., 2007; Zalloni et al., 2016). As for most xylem anatomical traits, IADFs are typically the result of trade-offs between competing demands for growth, stem support, carbon storage, and transport of water and nutrients under changing climatic conditions (Vaganov et al., 2006; Zweifel, 2006). Differences in tree size and age can also play a role to determine the resulting IADF features (Campelo et al., 2013, 2015). IADF formation is not necessary linked to bimodal growth pattern; for example, Rigling et al. (2003) found that IADF formation was driven by cool and moist summer conditions in dry Alpine sites. In eastern Spain, dry conditions induce a reduction of summer growth rate in Aleppo pine followed by a reactivation of cambial activity during rainy autumns which often leads to the formation of IADFs (de Luis et al., 2007, 2011, Camarero et al., 2010, Pacheco et al., 2016). Nevertheless, under highly variable climatic conditions and with the hysteresis of some growth processes, it is possible to have significant site- and species-specific effects (Novak et al., 2013a, 2013b; Battipaglia et al., 2016) together with significant delays between the triggering climatic factor and its translation into the wood anatomical imprints (Camarero et al., 1998).

Stem radial growth and xylem phenology and development (xylogenesis) are usually assessed with band or point dendrometers and repeated wood-sampling. Both these intensive monitoring approaches proved to be very effective to thoroughly investigate causal relationships between environmental variability and cambial dynamics across many different biomes and tree species (Rossi et al., 2016). However, most of these researches last for relatively short periods, often encompassing less than five years. For this, in areas with a significant year-to-year climate variability, the risk is to monitor beside growth, the seasonal stem-water content fluctuations combined with idiosyncratic site and climate conditions of the sampling years (Drew and Downes, 2009). This limitation in the temporal length of the investigations is very common in the drought-prone Mediterranean areas, where there are still very few studies on radial-increment dynamics longer than five years providing a thorough interpretation on climate-growth relationships (but see Ribas, 2006, Gutiérrez et al., 2011). This calls for a better understanding of the weather settings that cause IADF formation if we aim to use them as bimodality markers in Mediterranean tree species. To this end we proposed a different approach straggling between the typical short-term intensive monitoring adopted tracking cambial activity with xylogenesis or dendrometers monitoring and the long-term retrospective investigation applied with tree-ring analysis. Here we still consider dendrometer records but we adjoined this short-term information with dendroanatomical analysis to untangle the high-resolution but long-term information encoded in tree rings (Gartner et al., 2002; Fonti et al., 2010; von Arx and Carrer, 2014). A previous pilot investigation, applying this innovative dendroanatomical approach, has already successfully assessed intra-annual growth fluctuations in a long-term context. This helped to match short-term cambial activity monitoring with long-term (six decades) wood-anatomical time series and to improve the interpretation of the climate/growth relationships (Pacheco et al., 2016).

Aleppo pine (*Pinus halepensis* Mill.) is the most widespread tree species in the Mediterranean Basin lowlands (Ne'eman and Trabaud, 2000). It tolerates many soil types and grows under very diverse climatic conditions, from continental inland sites to mild conditions close to the coastal areas, but generally subjected to summer drought (Barbero et al., 1998). It is also a fire-adapted, shade-intolerant species that is able to withstand dry conditions (Borghetti et al., 1998). These characteristics make Aleppo pine the most suitable conifer tree species for studying the effects of summer drought within the context of climate change in the Mediterranean. We selected two Aleppo pine stands located in eastern Spain and subjected to contrasting Mediterranean climate types: a continental site located near Zaragoza and a mild coastal site near Barcelona. We expect that changes on stem diameter and wood-anatomical features (i.e. lumen area and cell wall thickness) will describe similar patterns showing the bimodal growth, but also to find differences in the response to seasonal changes in water availability between drier and wetter Mediterranean sites. We also hypothesize that any difference found in intensity of the bimodal growth patterns and frequency of IADFs between sites will be reflected at the wood anatomy level.

Our specific aims were (1) to describe and compare the intra-annual patterns of stem radial growth captured by each methodology in Aleppo pine at both study sites, (2) to assess whether the climatic signal in anatomical traits mirrors the bimodal growth pattern shown by the dendrometers, and (3) to determine which previous and intra-seasonal climatic conditions are triggering the formation of IADFs.

2. Materials and methods

2.1. Study sites and climate

The study was conducted in two locations with mild (wet) and continental (dry) Mediterranean climatic regimes situated in coastal and inland sites, respectively. For the coastal Mediterranean site, we selected an interior valley near Barcelona (northeastern Spain) on the Garraf karstic mountains (41° 20′ N, 1° 50′ E) in the central Mediterranean coastal ranges at an altitude of 300 m a.s.l. in the Natural Park of Garraf and Olèrdola. Vegetation cover is around 95% and is mainly comprised of *P. halepensis* with *Quercus coccifera* L. maquis dominating southern slopes and *Q. ilex* L. on the northern ones (Gutiérrez et al., 2011). The soil type is fersiallitic, decarbonated and argillic, which makes it susceptible to compaction. The presence of rocky outcrops creates a shallow and discontinuous soil surface with a low water-holding capacity (Ribas, 2006; Gutiérrez et al., 2011). According to climatic data from the nearest weather station at Begues (ca. 8 km from the study site), mean annual temperature for the period 1950–2014 was 13.2 °C with a maximum mean monthly temperature of 21.1 °C (July) and minimum of 5.1 °C (January). Mean annual precipitation was 673 ± 174 mm, with 33% recorded during autumn (September to November), 25% during spring (March and May) and an estimated water deficit period spanning from June to August (Gutiérrez et al., 2011) (Supplementary material, Fig. S1).

For the continental Mediterranean site we selected a mixed Mediterranean forest (Vedado de Peñaflor, 41° 47′ N, 0° 43′ W) near Zaragoza (Middle Ebro Basin, northeastern Spain) at an altitude of 340 m a.s.l. The terrain consists of small hills and valleys with west- and east-facing slopes, covered by a mixture of *P. halepensis*, *Juniperus phoenicea* L., *J. thurifera* L. and *Q. coccifera* L. among other woody species (Camarero et al., 2010). The soil at the site originates from a parent rock comprising gypsums and marls. Its depth is considered to be around 50 cm by observing trees uprooted by wind on the site (J.J. Camarero, personal observation). The climate, based on data from the nearest weather station at Aula Dei (ca. 5 km from site) during the period 1950 to 2014, is classified as continental Mediterranean with an average annual rainfall of 313 mm (semi-arid conditions). Annual mean temperature was 13.8 °C with frosts occurring from December to January and maximum and minimum mean monthly temperatures of 25.0 °C (July) and 6.4 °C (January), respectively. The period with water deficit spans from March to October (Supplementary material, Fig. S1).

2.2. Climate data

For both study sites there were weather stations nearby with fairly complete daily data available since 1952. For the Zaragoza site we used precipitation records from the Peñaflor-Aula Dei (41° 45′ N, 0° 49′ W, 280 m a.s.l.) and temperature records from Zaragoza-airport (41° 40′ N, 1° 00′ W, 263 m a.s.l.) weather station located at 30 km from the study site. At the Barcelona site we used all daily data from the Barcelona-airport (41° 17′ N, 2° 05′ E, 1 m a.s.l.) weather station, some 15 km to the east of the study site. We considered the daily-resolved records for mean, maximum and minimum temperatures, relative humidity, and total precipitation for the period 1952–2012. Vapour pressure deficit (VPD) was estimated using temperature and humidity data (Abtew and Melesse, 2013). We also calculated the water balance as the difference between precipitation and potential evapotranspiration (PET) following Hargreaves et al. (1982). The soil moisture deficit (SMD) was estimated as the difference between cumulative evapotranspiration and cumulative rainfall (Cocozza et al., 2012). Daily data were also averaged (temperatures) or summed (precipitation, water balance) at various time intervals (5, 10, 15, 20, 30, 40, 50 and 60 days) to analyze climate-growth relationships at multiple temporal scales following Gutiérrez et al. (2011).

2.3. Dendrometer measurements

Radial increment in Aleppo pine trees was monitored using stainless-steel manual band dendrometers (Agricultural Electronics, Tucson, USA). Dendrometers were placed at 1.3 m around the stem after brushing the dead bark. The site in Barcelona was divided into two groups of trees of different age. One group comprised 10 trees with an average age of 33 years and a measurement period extending from 1995 to 2004 (120 months), while a second group of 5 older trees (age = 75) was measured from 2001 to 2004 (48 months). In Zaragoza the period of data collection went from 2006 to 2014 (115 months) and was performed on one group of 10 trees with ages ranging from 35 to 75 years. For both sites dendrometer readings were taken up to twice per month during the growing season and main tree characteristics were measured at the beginning of the trial in Zaragoza (2006) and at the end in Barcelona (2004) (Supplementary material, Table S1). The cumulative circumference data, provided by the dendrometers, was converted to cumulative radial increment data assuming that the stem was cylindrical and dividing the girth data by 2π. For each tree, we calculated daily radial-increment rates by dividing the radial increment by the number of days between two consecutive observation dates.

2.4. Sample collection and wood-anatomy analyses

During the winters of 2014 and 2015, a total of 20 dominant trees (10 per site), from the same sites as the dendrometer trial, were randomly selected and sampled. At the Barcelona site the cores were taken from the older group including 4 trees with dendrometers, while in Zaragoza cored trees did not include those with dendrometers installed. We took three radial cores per tree at 1–1.3 m with Pressler increment borers 5 and 10-mm diameter. All cores were prepared following standard dendrochronological protocol (Stokes and Smiley, 1968). The ring widths were measured to the nearest 0.01 mm using LINTAB measurement equipment fitted with a stereoscope and TsapWin software (Rinntech, Heidelberg, Germany). To ensure correspondence between each annual ring and the calendar year of ring formation, cross-dating was checked using COFECHA software (Holmes, 1983). The 10-mm cores were then used for the histological analyses.

Cores selected for wood-anatomical analyses were divided into pieces 4-cm long, boiled in water to soften the wood and sliced into 10–12 μm thick transversal sections using a rotary microtome (Leica RM 2025, Heidelberg, Germany). The samples were stained with safranin (1%) and astrablue (0.5%) (both were diluted in distilled water) and rinsed with water and ethanol. They were then fixed on permanent slides with Eukitt (BiOptica, Milan, Italy) (von Arx et al., 2016). Histological images were obtained using a digital camera (Nikon Digital Sight DS-5M) mounted on a light microscope (Nikon Eclipse80i; Nikon, Tokyo, Japan); images were captured at 100× magnification. Overlapping images were taken from each sample and stitched together using the PTGui software (New House Internet Services, Rotterdam, NL) to obtain high-resolution images (0.833 pixels μm-1) of the entire section. Tree-ring borders were manually drawn on the images, which went through a semi-automatic analysis using the ROXAS software (von Arx et al., 2016). This analysis provided measurements (among others) on transversal lumen area (LA) and cell-wall thickness (CWT) (Prendin et al., 2017) of tracheids, and also gave the relative position of each tracheid within the dated annual ring.

2.5. Growth and wood-anatomy chronologies and relationships with climate

Chronologies of tree-ring width and wood-anatomical traits were produced for each site using ARSTAN. This software was developed to remove biologically induced age-size related growth trends (Cook, 1985), which are also present in wood-anatomical data (Carrer et al., 2015). Individual series were fitted using a cubic smoothing spline function with 50% frequency–response cutoff of 30 years. Subsequently, observed values were divided by expected values to obtain detrended and dimensionless indices, which were then subjected to autoregressive modelling to remove the remaining serial autocorrelation. These residual or pre-whitened indices were averaged using the biweight robust mean to create the site chronology of each variable for the two species.

Using the relative position of each tracheid within each annual ring, we calculated mean values of LA and CWT along 100 equal-width tangential sectors to obtain a very detailed profile of anatomical features changes along the whole ring. To assess the associations with climate, we simplified the 100 sector scheme by averaging it into a 10 sector scheme (i.e. 10 equal-width tangential sectors per ring). These "deciles" helped us to better define the time windows over the phases of radial growth. The climate-growth associations were quantified by calculating Pearson correlations between wood-anatomical detrended chronologies and the different time windows of climatic data for the best-replicated period (1950–2014).

2.6. IADF analyses

IADFs were firstly identified visually and we considered only those characterized as earlywood-like cells within the latewood, which were more abundant at both study sites. After this identification, we calculated the presence (frequency %) of IADFs per year for each site (Supplementary material, Fig. S3). The frequency of IADFs was assessed on a year basis as the percentage of trees showing a density fluctuation in any given ring. Then, to objectively compare sites we used the following protocol: i) we selected the years with the clearest single IADFs with at least a 50% frequency at site level, ii) using the minimum cell lumen area value of the density fluctuation as a center point, we split the tree ring profile into "pre-IADF" and "post-IADF" sectors, iii) we standardized the two portions of the profile using the tracheidogram approach (Cook and Kairiukstis, 1990; Vaganov et al., 2006). Lastly iv) we produced a mean tree-ring profile per site by averaging each year and tree profiles.

2.7. Growth and wood-anatomy statistical models

We applied Generalized Additive Mixed Models (GAMMs, Wood, 2006) to study the patterns in yearly radial increment data from dendrometers and wood cell formation from anatomical measurements. Generalized additive models are a semi parametric regression model that allows a flexible representation of the dependence of the response variable in the covariates by using a sum of smooth functions. The use of GAMMs makes it possible to model non-linear patterns in the response variable at the cost of increasing complexity in the model specification.

In this case the response variable was the growth rate according to the dendrometer measurements. Growth rate was modelled as a function of two covariates: one representing the cyclic duration of the year in days (day of year), the other the duration of the experiment in days (time).

$$\text{Growth rate}_i = \mathrm{f}_1(\text{day of year}_i) + \mathrm{f}_2(\text{between years trend}_i) + e_i \qquad (1)$$

where f_1 and f_2 represent smoothing functions of the two covariates and e_i random variable. We modelled the day of the year as a cyclic cubic regression spline in order to account for the cyclic behaviour of intra annual stem growth increments within a year and time as a cubic regression spline. In our case we used tree identity as random factor since each rate of growth represents repeated measurements from each tree. We also included an AR1 autocorrelation structure to control the cyclic variation of growth within each year.

A similar protocol was used to study the patterns in wood anatomical formation. In this case we modelled lumen area and cell wall thickness as a function of time and ring-wood sector.

$$\text{Cell size} = \mathrm{f}_1(\text{tree}-\text{ring section}_i) + \mathrm{f}_2(\text{time}_i) + e_i \qquad (2)$$

Each tree-ring was divided in 100 sections to represent the yearly variation in wood anatomy (tree ring section). We included tree as random factor in the analyses since measurements within each tree sample were repeated over time. We also included a correlation structure to control within tree-ring patterns in wood formation. A similar procedure was applied by Pellizzari et al. (2016). Statistical analysis, computing and graphics were performed using R (R Development Core Team, 2015).

3. Results

3.1. Radial increment analysis

Radial-increment dynamics in Aleppo pine featured a similar bimodal pattern in both sites with two main peaks during spring and autumn and very low increment rates during summer and winter (Fig. 1). The mild-wet site at Barcelona shows higher radial increment values for both growth peaks when compared to the fluctuations observed in the continental-dry Zaragoza site. The trough is instead very similar for both sites, although it occurs earlier in Barcelona (day 205 ± 0.2, 23 July) than in Zaragoza (day 230 ± 0.2, 17 August) ($t = -82.34$, $P < 0.001$) (Table 1). The growth peak in spring occurs almost simultaneously in both sites: Barcelona (133 ± 0.1, 12 May) and Zaragoza (134 ± 0.2, 13 May) ($t = -5.37$, $P = 0.01$). Instead the autumn growth season peaks slightly earlier in Zaragoza (276 ± 0.2, 2 October) than in Barcelona (280 ± 0.3, 6 October) ($t = 10.48$, $P = 0.001$).

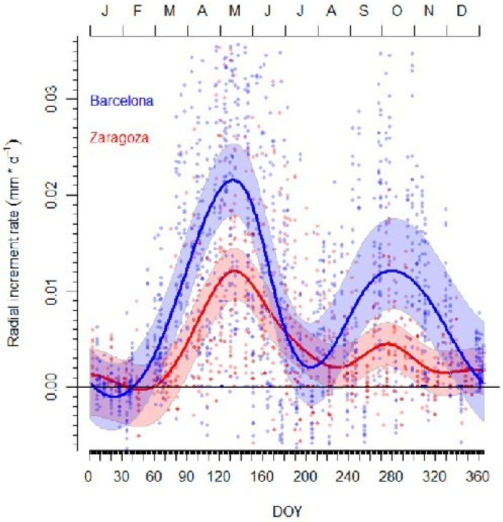

Fig. 1. Observed (points) and fitted (lines with confidence intervals) radial increment rates for the Aleppo pines in Zaragoza (red lines and symbols) and Barcelona (blue lines and symbols). The fitted lines highlight the growth bimodality and correspond to Generalized Additive Mixed Models (GAMMs). Portrayed data corresponds to the whole measurement period on each site.

3.2. Wood anatomy features and intra-annual growth patterns

In total, 1.29×10^6 and 1.25×10^6 tracheids were measured in the Zaragoza and Barcelona study sites, respectively. Based on these measurements we produced a very detailed quantitative description of Aleppo pine wood anatomy from 1950 to 2014 for both study sites (Fig. 2).

In the continental site, tracheids showed smaller LA (333 ± 11.5 μm^2) and CWT (4.05 ± 0.01 μm) than those from the coastal region in Barcelona (424 ± 18 μm^2 and 4.7 ± 0.03 μm, respectively). The changes in cell size followed similar intra-annual trends for LA at both study sites, but with a steeper decrease in size at the coastal site when approaching the latewood. This decrease is accompanied by a strong and clear increase of CWT (with the peak reaching >5.5 μm), which is much more subtle in the case of the continental site in Zaragoza. The main descriptive statistics commonly used in tree-ring research were computed to compare site wood anatomy chronologies and can be consulted in Supplementary material Table S2 and Fig. S2.

The trees in Barcelona produced more IADFs than those in Zaragoza (Table 2). Barcelona trees presented 27 years with IADFs frequencies of 50% or higher, against just 20 years for the Zaragoza pines. If we

Table 1
Estimated days of the year when the early and late growth peaks and the trough between peaks are predicted to occur based on radial increment rates (dendrometer data). Estimates correspond to Aleppo pines from the Barcelona (1995–2004 period) and Zaragoza (2006–2014 period) study sites. Values are means ± SE and differences between sites were assessed using *t*-tests. The estimated days were obtained by fitting Generalized Additive Mixed Models (GAMMs) to radial increment rates.

Periods of radial increment	Barcelona	Zaragoza	*t* (*P*)
Early peak	133 ± 0.1	134 ± 0.2	−5.37 (0.01)
Trough	205 ± 0.2	230 ± 0.2	−82.34 (<0.001)
Late peak	280 ± 0.3	276 ± 0.2	10.48 (0.001)

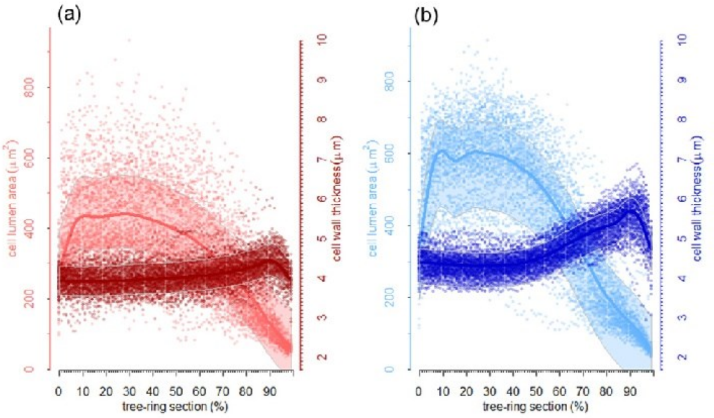

Fig. 2. Observed (points) and fitted (lines with confidence intervals) tree-ring wood-anatomical profiles for cell lumen area (light colours) and cell wall thickness (dark colours) of Aleppo pines in Zaragoza (a) and Barcelona (b). The fits highlight the intra-annual trends in tracheid lumen area and wall thickness and correspond to Generalized Additive Mixed Models (GAMMs). Data correspond to the common 1950–2014 period.

consider instead the years with total absence of IADF (i.e. a 0% frequency in IADF presence), the difference between sites becomes more evident: Barcelona had just 8 years without IADF, whereas in Zaragoza there were 23 years without them.

Selecting the years with a 50% or higher frequency of IADF (i.e. a single and clear density fluctuation per ring) it can be observed that the minimum LA within the IADF is located almost in the same sector for both sites (Zaragoza: sector 76 ± 9.2, and Barcelona: sector 74 ± 7.7), but with different characteristics (Fig. 3). While in a typical IADF year at Zaragoza the earlywood cells LA follows the main trend very closely, the Barcelona earlywood cells are well above the LA average, although still within the GAMMs confidence intervals of all the studied years. A contrasting behaviour is observed during the ring section corresponding to the IADF. The LA values of Zaragoza trees show a continuous decrease that passes below the lower limit of the GAMMs confidence intervals. Then, in the recovery phase the earlywood-like tracheids never regain average values. In the case of Barcelona, after a steep decrease in LA to reach the minimum point of the density fluctuation, it barely skirts the expected trend of the global GAMMs confidence limits, and LA values rise back to the average trend in the recovery phase.

3.3. Climate-wood anatomy relationships

Considering that water availability is one of the main drivers of forest productivity and wood formation in Mediterranean sites, we calculated Pearson correlations for different weather parameters related to precipitation. The most robust and significant correlations were obtained using 40-day moving windows and accumulated soil moisture deficit (SMD). Results show clearly the importance of SMD for the enlargement of LA in tracheids of Zaragoza Aleppo pines. The significant positive correlation in Zaragoza extends throughout the growing season, even before its start for the first half of the sectors (Fig. 4), while for the second half, a clear peak of positive correlations can be observed during early summer (late June). In the case of Barcelona, only the first four sectors have a slight positive correlation mostly during late spring and early summer.

Table 2
Comparison of the amount of years with intra annual density fluctuations (IADF) occurrence, considering two frequency ranges: above and below 50%, plus two absolute categories: full presence (100%) and total absence (0%), for both sites.

Frequency	Barcelona	Zaragoza
≥50%	27	20
<50%	36	43
100%[a]	8	3
0%[a]	8	23

[a] Values already accounted on the two first categories.

The analysis of precipitation regimes, considering the years when IADF were observed separately from those when they were absent, showed differences in both sites (Fig. 5). In Zaragoza, the years without IADFs present almost no difference from the mean average rainfall recorded during the study period (1952–2012). Instead, the IADF years have a much rainier late summer and early autumn. In addition, these IADF years have drier conditions at the beginning of the growing season (late March and early April). At the Barcelona study site the winter months (January and February) are drier than the average for IADF years, while autumn rains (beginning of September) come around 40 days earlier in IADF years than non-IADF years.

4. Discussion

In this study, the combined use of high-frequency but rather short-term dendrometers records with long-term wood anatomy analysis allowed us to assess the bimodal growth of *Pinus halepensis* where the xylem anatomical traits reflect the climatic conditions driving xylogenesis. We selected Aleppo pine for its wide distribution around the Mediterranean Basin. This allowed us to compare its growth pattern between two different Mediterranean climatic regimes (coastal and continental). This comparison showed us how in wetter and milder coastal conditions the species presents a more pronounced bimodal growth, larger cells and higher frequency of IADFs than on the dry continental site.

In Mediterranean climates, many tree species have a growing season with two distinct growth peaks and a low growth activity period in between (Liphschitz and Lev-Yadun, 1986). This bimodal xylogenesis behaviour is also associated with the formation of intra-annual density fluctuations (IADF) and has been reported in several conifers (*Pinus halepensis*, Ribas, 2006, Camarero et al., 2010, Touchan et al., 2012, de Luis et al., 2007, 2011; *Pinus pinaster*, Rozas et al., 2011, Vieira et al., 2014, Carvalho et al., 2015; *Juniperus thurifera*, Olano et al., 2012, Pacheco et al., 2016; *Pinus pinea*, Campelo et al., 2007) and hardwood tree species (*Quercus ilex*, Gutiérrez et al., 2011; *Arbutus unedo*, Battipaglia et al., 2010, De Micco et al., 2012).

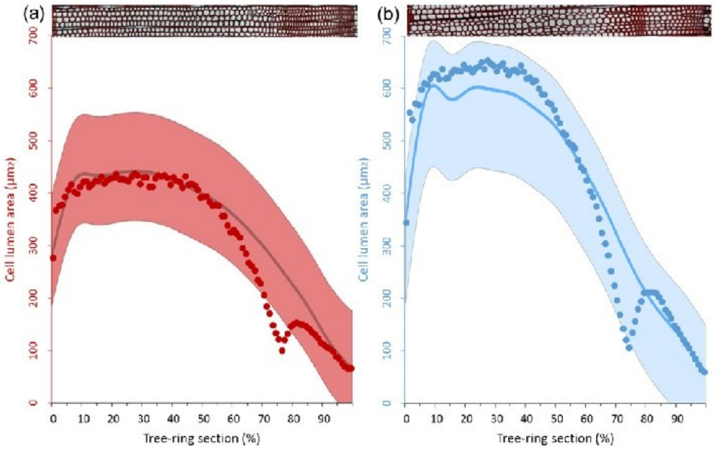

Fig. 3. Mean tracheid lumen area profiles (red and blue dots) for the intra-annual density fluctuations (IADF) years within the 1950–2014 period of Aleppo pines from Zaragoza (a) and Barcelona (b). Shadings and lines represent the global GAMMs fitting as in Fig. 2. The top images show two typical rings with IADFs.

Our nine and ten-year long record of radial-increment measurements portrays, in concordance with other Mediterranean conifers, a very similar bimodal pattern of growth for both sites and ages (Campelo et al., 2013; Vieira et al., 2013, 2015). The two age groups in the Barcelona site highlighted no differences on the bimodal growth pattern except for the expected distinct growth rate (Supplementary material, Fig. S5). The results from the GAMMs (Fig. 1) show how this bimodality in growth rates is more consistent in Barcelona, where precipitation is higher, the weather milder and growing seasons last longer. Trees in both sites experience a similar period of low growth activity during the dry summer, but while the two peaks of high activity coincide almost perfectly in time, the trough shows a delay of almost one month in Zaragoza (Table 1). When we overlapped these periods of minimal growth activity with the average precipitation records (Fig. 5), it emerges that the trough matches with the driest days of the year for both sites (i.e. end July in Barcelona and mid-August in Zaragoza). Given that the precipitation average was calculated over a period of 60 years and matched our radial increment results, we consider that a 10-year long dendrometer record might be considered sufficient to obtain reliable stem growth data in areas with strong inter-annual weather variability.

One of the main differences between the climates at both sites is the actual amount of soil water trees may use, i.e. considering the lower rainfall and higher evapotranspiration rate in Zaragoza as compared with Barcelona, and how it is seasonally distributed. Our results on wood-anatomical traits show how Aleppo pines produce tracheids with smaller lumen area in the semi-arid Zaragoza and bigger ones in the more mesic coastal Barcelona (Fig. 2), where rainfall is twice that in the inland site (Supplementary material, Fig. S1). These intrinsic climatic conditions also result in taller, even though younger, trees at the Barcelona site (Supplementary material, Table S1) which, considering the mechanistic link between conduit-lumen size, tree architecture and height growth (Carrer et al., 2015), also accounts for the difference in mean cell area between sites (Fig. 2). It is known that cambial activity

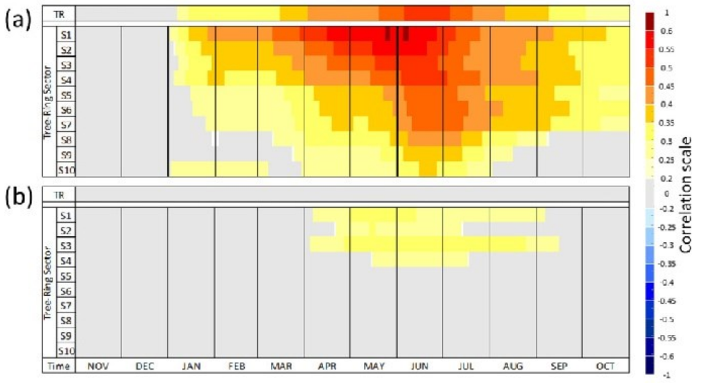

Fig. 4. Climate-growth associations comparing lumen area with soil moisture deficit in Aleppo pine for both study sites (a, Zaragoza; b, Barcelona) and over the 1952–2012 period. Correlations were computed from November of the previous year to the following October considering each tree-ring sector separately from the earlywood (S1) to the end (S10) of the ring. Correlation coefficient above |0.254| is significant at $P < 0.05$.

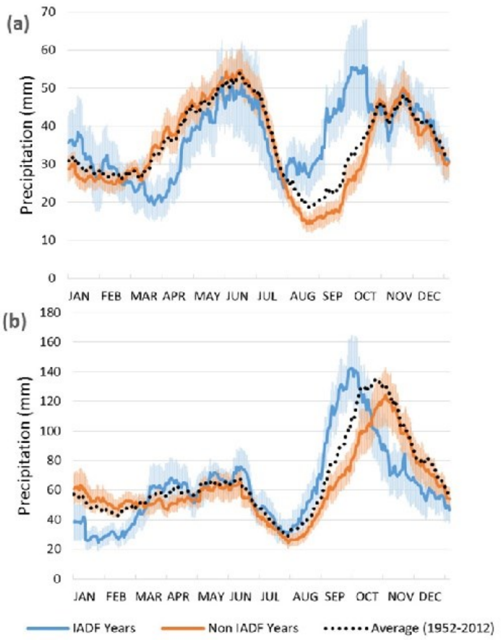

Fig. 5. Rainfall patterns of the years with high (>50%) and low (<50%) frequencies of intra-annual density fluctuations (IADFs) for the two study sites (a, Zaragoza; b, Barcelona) in the 1952–2012 period. Rainfall values are calculated as 40-days running-windows; coloured bars indicate standard errors of each value.

in a Mediterranean climate is controlled principally by water availability during the growing season (Cherubini et al., 2003) and variations on this availability can affect the tracheids anatomical features (Pacheco et al., 2016). As mentioned before even though both sites follow the bi-seasonal rainfall pattern, Zaragoza has its highest precipitation peak during spring, while Barcelona experiences it during autumn and maintains an almost uniform precipitation from winter to the spring rainfall peak. This steady availability of water before and during the first part of the growing season explains the better overall growth and therefore bigger tracheids size in the Barcelona pines. Transversal lumen size in tracheids plays an important role in the trade-off between hydraulic conductivity and vulnerability to cavitation (Willson and Jackson, 2006, Hacke, 2015), and is strongly related to water availability during the phase of tracheid radial enlargement (Vaganov et al., 2006; Vieira et al., 2009; DeSoto et al., 2011). In Zaragoza the scarcity of precipitation before the growing season and the relatively lower spring rain peak (if compared with Barcelona) is probably a contributing factor to the smaller cell size of its pines.

The presence of IADF occurs mainly in the last third of the tree ring profile (Vieira et al., 2010, Rozas et al., 2011, Novak et al. 2013). Our samples show that the sharp reduction in lumen area corresponding to the IADF occurs at 0.75 of the distance from the ring border (i.e. sector 75 ± 1 of the tree-ring profile), and both sites present similar minimum values of cell area (Fig. 3). This can be interpreted as trees in both sites following a similar process in the formation of summer latewood. When they enter the recovery phase (i.e. cambium reactivation), tracheids in Barcelona display lumen areas 50% bigger than Zaragoza trees, and while the first ones reach the average expected values of their predicted tree-ring profile, those from Zaragoza stay well below theirs. These long-term results complement those obtained from the dendrometer analysis, showing a stronger bimodal plasticity not just during regular years but also affecting cell production during the years with IADF formation.

Soil water availability is the environmental driver of tree growth and wood anatomy with the most striking contrast between sites. In the one hand, xeric characteristics at Zaragoza are probably the reason why lumen area positively correlates so significantly with soil moisture deficit. On the other, pines in the wetter Barcelona show a slight response to this parameter, as they grow under less limiting water conditions. Separating IADF and non-IADF years in Zaragoza, significant positive values are maintained in the non-IADF years, but they almost disappear during the years when IADFs are formed (Supplementary material, Fig. S4). This may represent an indicator that IADF are formed when soil water availability is scarce, and therefore the trees show a stronger response to late-summer water availability that otherwise would not trigger the formation of an earlywood-like IADF. As seen in Fig. 5, the years with IADF formation registered a significant drop in precipitation a couple of weeks before and during spring cambial activation (i.e. late March for Zaragoza and February in Barcelona). This condition, together with the precipitations in late summer and early autumn, is a clear example of the complexity of factors contributing to wood formation and its intra-annual features.

5. Conclusions

In conclusion, we found that growth bimodality and IADF formation, two facets of the double period of radial growth and wood formation in Mediterranean forests, were more pronounced in the mild-wet site (Barcelona) than in the cold-dry site (Zaragoza), which also displayed a shorter growing season. In the drier site, tracheid lumen area responded to short-term (weekly scales) changes in precipitation and soil water availability during the period when growth rates peaked, i.e. late spring (May) to early summer (June) (compare Figs. 1 and 4). Furthermore, trees in the wetter Barcelona site formed wider lumen areas, which in addition to the longer growing season, could explain why they also presented higher growth rates, more IADFs and a more pronounced bimodal behaviour.

Matching short-term intensive monitoring with long-term retrospective wood anatomy records proved to be an efficient combination for a quantitative and robust assessment of cambial phenology (Carrer et al., 2017), IADFs (Battipaglia et al., 2016) and to define the climate drivers triggering the bimodal growth pattern in Mediterranean environment.

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Conflict of interest

None declared.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2017.09.133.

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