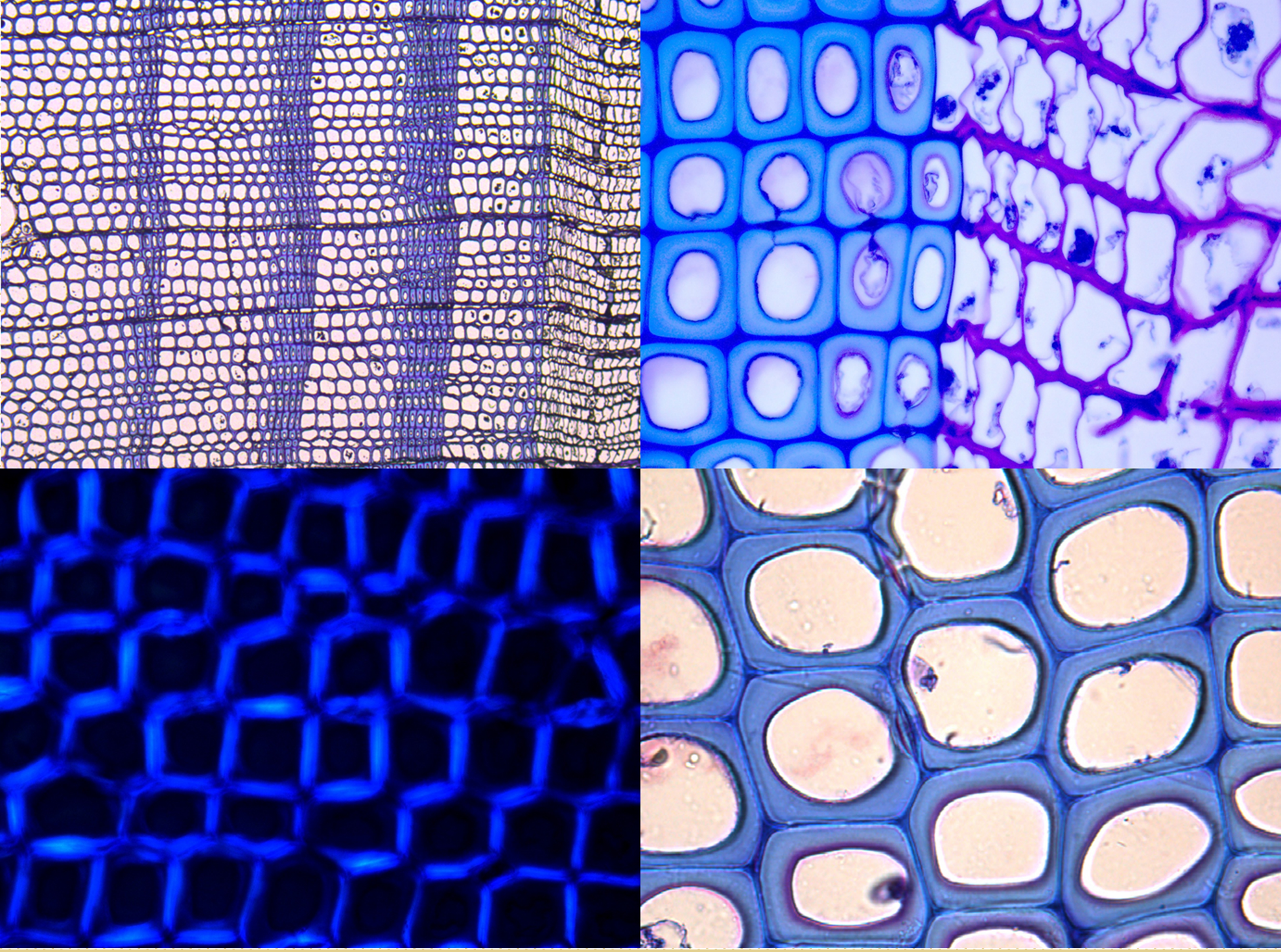

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Cambial activity and wood formation of maritime pine in a drought-prone environment: the effect of growth rate, size and climate

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Cambial activity and wood formation of Maritime pine in a drought-prone environment: the effect of growth rate, size and climate

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List of publications

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Vieira J, Rossi S, Campelo F, Nabais C (Accepted for publication) Are neighbouring trees in tune? – Wood formation in *Pinus pinaster*. European Journal of Forest Research.

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Abstract

The formation of tree rings is a slow and complex process. The year-to-year climatic variability and the constant interaction between the internal and external factors controlling cambial activity, create the conditions that make each tree ring unique. In order to capture the dynamics of cambial activity and wood formation during the growing season, it is necessary to monitor wood development in narrower time intervals (from minutes to weeks). Most of the studies on cambial activity and wood formation were held in cold environments, but in other environments, such as drought-prone areas, still remains poorly understood. In order to understand the cambial activity and wood formation under Mediterranean climate, a drought-prone environment, timing and dynamics of cambial activity in maritime pine (*Pinus pinaster* Ait.) were monitored during two years (2010 and 2011). Anatomical observations of the cambial zone and differentiating xylem were made and stem radial increment monitored using manual and automatic dendrometers. The studies described in this thesis were carried out in Perimetro Florestal Dunas de Cantanhede, a managed plantation of maritime pine located in the west coast of Portugal.

The cambial activity and wood formation of maritime pine trees with the same age and size but different growth rates in the period 2009-1994 (classified as fast and slow trees), was monitored throughout 2010, to determine whether the observed differences in tree-ring width were triggered by the timing of cambial activity or by the rate of cell production. It was determined that the timing of cambial activity was similar in both growth rate classes. However, fast-growing trees presented higher rates of rate of cell production than slow-growing trees. The band dendrometer readings revealed a bimodal pattern of stem radial increment, with two peaks of increment, one more pronounced in spring and another in autumn. Although the bimodal pattern is typical of trees growing in the Mediterranean region, the combined analysis of anatomical observations of the cambial region and band dendrometers showed that the second period of radial increment corresponded mostly to the re-hydration of the stem, since no resumption of cambial activity was observed in autumn.

In order to determine if differences in stem diameter were due to different rates of cell production or xylogenesis timings, the cambial activity of even-aged trees belonging to two diameter classes was monitored throughout 2011. The timings of cambial onset and differentiation were the same in both diameter classes. However, enlargement and cell wall deposition lasted longer in large trees. Besides the different durations, large trees also showed a higher rate of cell production. Thus, revealing that the differences in diameter observed between the trees were due to the rates of cell production. In both diameter classes, the cambium was active from March to July, and quiescent from August to November, suggesting that in the Mediterranean region, trees are under a double climatic control: low temperatures and reduced photoperiod in the winter and high temperatures associated with low water availability in the summer. Summer quiescence was broken in late October, when precipitation re-hydrated the stem. In November, cambial divisions were observed, indicating that maritime pine has the ability to form new xylem cells after the summer drought.

The influence of climate on the cambial activity and wood formation of maritime pine was studied over two dry years (2010 and 2011). It was found that cambial onset started earlier in response to a warmer late-winter and stopped earlier in response to a drier spring and summer, confirming that Mediterranean conifers are under a double climatic control. Low water availability during spring and summer limited cell production, which affected tree-ring width. Drier conditions also triggered an earlier start of latewood formation, leading to the development of fewer tracheids with smaller lumen area. It was also observed that the duration of xylogenesis was not dependent on cambial onset. In fact, an earlier onset of xylogenesis did not trigger a longer duration of cambial activity.

To ascertain the influence of water availability on stem radial increment of maritime pine, hourly variations of stem radial increment and tree water deficit were monitored throughout 2010 using automatic dendrometers. The seasonal cycle was divided in five periods of distinct physiological activity: winter dormancy, spring growth, pre-summer contraction, summer quiescence and autumn re-hydration. The stem cycle approach was then used to divide the daily cycles in contraction, recovery and increment phases. Continuous positive radial increment started in spring and reached its maximum by the end of June, time at which a shrinking period was observed. The stem contraction observed in June was due to the inability of trees to recover the water lost by transpiration, contracting from one cycle to the next. In autumn, a period of re-hydration and rapid expansion was observed after precipitation. Daily variations in stem radius of maritime pine were mainly determined by the course of transpiration and thus, highly dependent on temperature and water availability.

Overall, the results obtained in this dissertation provided a detailed insight on the dynamics of maritime pine cambial activity in a drought-prone environment, the Mediterranean region. It was observed that the cell production rate was the main responsible for the differences in tree-ring width and ultimately in stem diameter. Within an even-aged and managed forest, different individuals can present different cellular production rates (fast and slow trees) that in time will be translated in different stem diameters (larger and smaller trees). Growth onset was not influenced by the size of the tree, but a longer duration of wood formation was observed in fast-growing and larger trees. In both years (2010 and 2011), the radial increment of all studied trees presented a clear bimodal pattern, with two increment peaks, as observed in other Mediterranean species. The first and more pronounced peak occurred in spring and a second less pronounced peak in autumn. The second growth peak corresponded mainly to a re-hydration of the stem after the summer drought. Climate played an important role in maritime pine cambial activity and wood formation, low temperatures and reduced photoperiod in winter and high temperatures associated with low water availability in the summer limited tree growth by imposing a dormant period.

Resumo

A formação de aneís de crescimento é um processo lento e complexo. A variabilidade climática interanual e a interacção entre os factores internos e externos que regulam a actividade cambial são os grandes responsáveis pela singularidade de cada anel de crescimento. De maneira a capturar a dinâmica da actividade cambial e da formação de madeira ao longo do período de crescimento, é necessária a sua monitorização ao longo do ano e a uma escala temporal reduzida (de minutos a semanas). A grande maioria dos estudos sobre actividade cambial e formação de madeira foi realizada em ambientes limitados pela temperatura. Noutros ambientes, e em especial naqueles limitados por condições de seca, continua a ser um assunto pouco estudado. Com o intuito de melhor caracterizar a actividade cambial e a formação de madeira na região Mediterrânica, a actividade cambial do pinheiro-bravo (*Pinus pinaster* Ait.) foi monitorizada ao longo de dois anos (2010 e 2011). Para tal, foram feitas observações anatómicas da zona cambial e xilema em desenvolvimento, e monitorizado o incremento radial do tronco através de dendrómetos manuais e automáticos. Os estudos descritos nesta tese foram realizados no Perímetros Florestal Dunas de Cantanhede, uma plantação gerida de pinheiro-bravo, localizada na costa Oeste Portuguesa.

A actividade cambial e a formação de madeira em pinheiros-bravos de idade e tamanho semelhantes, mas taxas de crescimento distintas entre 1994 e 2009 (árvores de crescimento rápido e lento), foi seguida ao longo do ano de 2010, com o objectivo de determinar se as diferenças observadas no tamanho dos anéis de crescimento foram devidas a diferenças no período de actividade cambial ou na taxa de divisão celular. O início da actividade cambial ocorreu simultâneamente em ambas as classes de crescimento. Contudo, as árvores de crescimento rápido apresentaram taxas de divisão celular maiores. As variações do diâmetro do tronco revelaram um padrão de crescimento bimodal, composto por dois picos de incremento, um de maior intensidade na Primavera e outro no Outono. Apesar de o padrão de crescimento bimodal ser característico da região Mediterrânica, a análise conjunta das observações anatómicas e das curvas de incremento do tronco revelaram que o segundo período de incremento correspondeu maioritariamente à re-hidratação do tronco, e não à reactivação da actividade cambial.

Com o objectivo de determinar se diferenças no diâmetro do tronco foram devidas a diferentes taxas de divisão celular ou a diferenças no perído de xilogénese, a actividade cambial de árvores com idade idêntica, mas com troncos de diâmetro diferente, foi seguida ao longo de 2011. O início da actividade cambial e diferenciação de xilema ocorreu simultaneamente em ambas as classes de diâmetro. Contudo, as fases de expansão e de deposição da parede secundária apresentaram uma maior duração nas árvores de maior diâmetro. Foi ainda observado que as árvores de maior diâmetro apresentaram uma maior taxa de divisão celular. Assim, foi concluído que as diferenças de diâmetro do tronco observadas em árvores da mesma idade foram devidas diferentes taxas de divisão celular. Em ambas as classes de diâmetro o câmbio permaneceu activo de Março a Julho, e quiescente de Agosto a Novembro. O período de actividade cambial descrito no pinheiro-bravo sugere um duplo controlo climático na região Mediterrânica: temperaturas baixas e fotoperíodo curto no Inverno, e temperaturas elevadas e baixa disponibilidade hídrica no Verão. A quiescência de Verão terminou após as primeiras chuvas após a seca de Verão (Outubro), que causaram a re-hidratação do tronco. Foram ainda observadas divisões celulares no câmbio em Novembro, o que é indicativo da capacidade do pinheiro bravo em produzir novas células de xilema após a seca de Verão.

A influência do clima, e em especial da disponibilidade hídrica, na actividade cambial do pinheiro bravo foi estudada ao longo de 2 anos (2010 e 2011). Foi determinado que um início precoce da actividade cambial estava relacionado com temperaturas mais amenas no final do Inverno. Observou-se ainda que o final precoce da actividade cambial estava relacionado com um Verão quente e com menor disponibilidade hídrica, confirmando um duplo controlo climático no crescimento das árvores. A baixa disponibilidade hídrica registada na Primavera e no Verão afectou negativamente o tamanho do anel de crescimento, promovendo o início precoce da formação do lenho tardio. Os traqueídos do lenho tardio, além de em menor número, apresentaram também uma menor área do lúmen. Observou-se ainda que o início e a duração da actividade cambial são independentes, uma vez que um início precoce não se traduziu num maior período de actividade cambial.

Finalmente, para determinar o efeito da disponibilidade hídrica nos ciclos diários e sazonais de incremento radial do tronco de pinheiro-bravo, a variação horária do incremento radial e o défice de água no tronco foram monitorizados ao longo de 2010. O incremento radial do tronco foi dividido em cinco períodos distintos de actividade fisiológica da árvore: dormência de Inverno, crescimento primaveril, contracção antes do Verão, quiescência de Verão e re-hidratação de Outono. Estes períodos foram estudados individualmente com o intuito de perceber as variações na amplitude e na duração das várias fases do ciclo diário de incremento radial do tronco ao longo do ano. O ciclo diário foi dividido nas fases de contracção, recuperação e incremento. Um período contínuo de incremento radial positivo foi observado durante a Primavera, atingindo o máximo em Junho. Este máximo foi seguido de um período de contracção, devido à incapacidade das árvores em recuperar a água perdida por transpiração durante o dia. O tronco das árvores foi contraindo até a árvore entrar em quiescência. A re-hidratação e expansão do tronco foi observada após as primeiras chuvas, no Outono. As variações diárias do diâmetro do tronco de pinheiro-bravo foram fundamentalmente devidas à transpiração e por isso dependentes da temperatura e da disponibilidade hídrica.

Os resultados da presente tese forneceram informação detalhada sobre a dinâmica cambial de pinheiro-bravo proveniente de uma zona caracterizada por um período de seca sazonal, a região Mediterrânica. Foi observado que a taxa de divisão celular foi a principal responsável pelas diferenças encontradas no tamanho dos anéis anuais de crescimento, culminando em diferenças significativas no diâmetro do tronco de árvores de idade semelhante. Numa floresta gerida com árvores de idade semelhante, é possível encontrar indivíduos com taxas de divisão celular diferentes (crescimento rápido e lento), e que com o passar do tempo se irão acumular e expressar como diferenças de diâmetro (árvores de maior e menor diâmetro). Foi concluído que o início da actividade cambial foi independente do tamanho da árvore, contudo uma maior duração da formação de madeira foi observada nas árvores de maior diâmetro e com taxas de crescimento rápidas. O incremento radial de todas as árvores estudadas em 2010 e 2011 apresentou um padrão bimodal típico da região Mediterrânica, com dois picos de incremento. O primeiro pico foi mais pronunciado e ocorreu na Primavera, enquanto que o segundo pico, menos pronunciado, foi observado no Outono. O segundo pico de incremento radial correspondeu à re-hidratação do tronco após a seca de Verão. O clima desempenhou também um papel importante na formação de madeira, com as temperaturas baixas e fotoperíodo curto durante o Inverno, e as temperaturas elevadas e baixa disponibilidade hídrica no Verão, a imporem períodos de dormência cambial.

CHAPTER I

General introduction

1.1 Dendrochronology

Trees, like all living organisms, respond to the changing environmental conditions with physiological regulations and cambial activity adjustments. Trees continuously adjust the timings of their growing activity in relation to the prevalent climatic conditions, which is reflected in the amount and characteristics of the xylem cells (Fritts 1976). Thus, the climatic conditions are imprinted in the wood making tree rings an excellent proxy of climate. Several climatic reconstructions have been made worldwide using tree rings. For example: Briffa *et al.* (1990) reconstructed the summer (April-August) temperature of northern Fennoscandia for the last 1400 years using tree-ring data, and Touchan *et al.* (2005) the May-August precipitation in the Eastern Mediterranean for the last 600 years. Tree-ring width can also be used to reconstruct drought indices: Nicault *et al.* (2008) reconstructed the Palmer Drought Severity Index in the Mediterranean region, which allowed them to identify the frequency and intensity of summer droughts in the last 500 years. These studies have allowed researchers to reconstruct the earth climatic conditions in the last millennium and compare past climatic trends with the present ones.

In addition to tree-ring width, other characteristics can be used to extract climatic information. Maximum latewood density was used to reconstruct the summer temperature in Alaska in the last nine centuries (Anchukaitis *et al.* 2012). In another similar study also using maximum density, Dorado-Liñán *et al.* (2012) reconstructed the May-September mean temperature in the Pyrenees Mountains of the last 750 years. Another tree ring characteristic frequently used in climatic reconstructions is isotope ratios. Treydte *et al.* (2006) used the oxygen isotope ratio of tree-rings cellulose to reconstruct the last millennium precipitation in Pakistan. In order to improve the climatic signal and reinforce the reconstruction, different tree ring features can be used together. McCarroll *et al.* (2013) used a combination of nine tree growth proxies to reconstruct the summer temperature of the last 1200 years in northern Europe and Russia.

Although dendrochronological studies are present worldwide, the climatic signal captured in tree rings is dependent on local climatic conditions. Trees at high latitudes and altitudes are mostly dependent on temperature, whereas trees from temperate climates and low altitudes are dependent on a combination of temperature and water availability (Vaganov *et al.* 2005; Linares *et al.* 2009). In the Mediterranean, tree growth is mainly dependent on water availability (Battipaglia *et al.* 2009; Gómez-Aparicio *et al.* 2011). Several dendrochronological studies on typical Mediterranean tree species have revealed that trees responded positively to spring precipitation, presenting wider tree rings in years with higher precipitation (Martín-Benito *et al.* 2008; Campelo *et al.* 2009; Vieira *et al.* 2009).

Wood formation is a slow and complex process and the annual resolution of tree-rings cannot fully describe the climate-growth relationship. In order to improve the time resolution of dendrochronological studies, intra-annual features such as earlywood and latewood widths (Lebourgeois 2000; Vieira *et al.* 2009), intra-annual density fluctuations (IADFs; Rigling *et al.* 2002; Campelo *et al.* 2007; Vieira *et al.* 2010), density profiles (Bouriaud *et al.* 2005; Martinez-Meier *et al.* 2009) and vessel features (García-González and Fonti 2006; Campelo *et al.* 2010), have been incorporated in dendrochronological studies. Fonti and Garcia-Gonzalez (2008) found a clearer climatic signal in earlywood mean vessel size than in tree-ring width chronologies of mesic site oaks. Anatomical structures such as IADFs are formed in response to specific climatic conditions, thus their presence and location within a tree ring provides valuable information on the climatic conditions recorded during the growing season (Campelo *et al.* 2007). Vieira *et al.* (2010) observed that latewood IADFs in maritime pine trees were positively correlated to September and October precipitation and that chronologies of latewood IADFs could be used to reconstruct autumn precipitation.

Although the inclusion of intra-ring features significantly increases the time resolution of tree rings climatic signal, all these assumptions were made retrospectively. To better understand the mechanisms and dynamics of wood formation and to assess the climatic influence on radial growth, wood formation needs to be continuously monitored.

1.2 The vascular cambium and the formation of tree rings

Tree rings derive from a lateral meristem: the vascular cambium, hereafter referred to as cambium. The meristematic cells of the cambium exist in two forms: fusiform initials, that are vertically orientated, and ray initials, which are horizontally orientated (Figure 1.1; Larson 1994). Secondary xylem and phloem are produced through periclinal divisions of the fusiform initials (Figure 1.1; Lachaud *et al.* 1999). When the derivative of the cambial initial is produced towards the outside of the root or stem, it becomes a phloem cell, but when it is produced towards the inside, it becomes a xylem cell. The ray initials produce horizontally orientated ray cells, which form the vascular rays or radial system (Larson 1994). The radial system is largely composed of parenchyma cells which serve as pathways for the movement of nutrients, carbohydrates and water between the secondary phloem and the secondary xylem, and also serves as a storage center (Raven 1999).

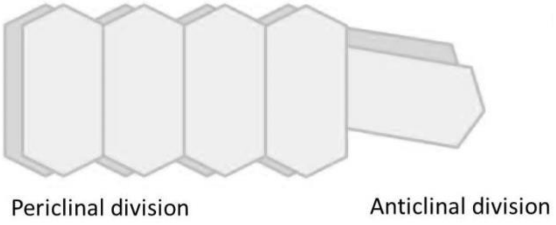

Figure 1.1 Periclinal and anticlinal divisions of fusiform and ray initials. Periclinal divisions are observed in the fusiform initials and are responsible for the formation of secondary xylem and phloem. Anticlinal divisions are observed in the ray initials and are responsible for the formation of the radial system.

Cambial activity is regulated by both internal factors, such as hormones, and external factors, such as photoperiod, temperature and rainfall. Auxin (indole-3-acetic acid) is often considered as the main phytohormone involved in the regulation of cambial activity (Larson 1994; Uggla *et al.* 1998; Lachaud *et al.* 1999). Cambium activation and growth follow a distinct polar direction, starting on the top of the plant, near the leaves, and slowly descend through the stem. This is due to the basipetal auxin flux (Uggla *et al.* 1996; Schrader *et al.* 2003). Auxin plays an important role in most aspects of secondary growth, such as cell division, secondary wall thickness and final size of xylem cells (Uggla *et al.* 1996). It also favors the formation of fusiform initials rather than ray initials (Lachaud *et al.* 1999). However, auxin is not the only phytohormone that regulates cambial activity. Gibberllins act directly on cambial cell division by changing the auxin levels, cytokinins promote an increase in the cambial sensitivity to auxin and ethylene has been found in higher concentrations when the cambial zone is active (Lachaud *et al.* 1999).

The annual course of cambial activity is strongly regulated by the climatic conditions, with cold or dry seasons imposing cambial dormancy, while warmer or rainy seasons inducing cambial activity (Larson 1994). The year-to-year variations in climate influence the quantity and quality of the wood formed. Thus, biological and physiological aspects of the regulation of cambial activity in trees are also of economic interest. The seasonality of cambial activity plays an important role in wood formation and reflects the adaptability of trees to their environment. In temperate and cold regions, cambium is dormant during the winter and active in spring and summer. Low temperatures and decreasing photoperiod in winter trigger trees to enter in dormancy. The break of winter dormancy has been largely studied, and it has been concluded that temperature plays a key role (Oribe *et al.* 2001; Begum *et al.* 2007; Rossi *et al.* 2008; Begum *et al.* 2010). Earlier onsets of cambial activity have been related to warmer spring temperatures (Rossi *et al.* 2008). Also, a relation between the end of cell division and temperature has been established in trees of cold environments, in order to guaranty that lignification is complete before winter (Gricar *et al.* 2005; Rossi *et al.* 2006).

Other factors such as age, size and dominance class of the trees also influence the timings and rates of cambial activity (Rossi *et al.* 2008; Rathgeber *et al.* 2011). Rossi *et al.* (2008) determined that older trees presented shorter and delayed periods of cambial activity and Rathgeber *et al.* (2011) observed that dominant trees exhibited a longer and more intense period of wood formation. A higher cell production rate has also been associated with longer durations of xylogenesis (Lupi *et al.* 2010; Rossi *et al.* 2012). However, similar observations were made in trees that started to grow earlier. Thus, there is a relationship between timings, growth rate and cell production, with both timings and rates responsible for a prolonged duration and higher cell production.

The seasonal cycles of cambial dormancy and activity are well described in trees of cold environments. However, seasonal dynamics of cambial activity and wood formation in trees from drought-prone environments is still fragmentary. It is thus important to consolidate the knowledge on xylem formation on such environments.

1.3 Xylogenesis

Cambium is generally defined as one layer of meristematic cells with the ability of an unlimited number of divisions (Larson 1994). During the growing season, cambium will derive some layers of cells with limited capacity of division: the xylem and phloem mother cells. The area where these cells divide is called cambial zone and contains the cambium and the xylem and phloem mother cells (Figure 1.2-a). After losing its ability to divide, the cambium derivative will be differentiated into xylem or phloem (Figure 1.2-b). The process of xylem differentiation starts with the enlarging of the cambium derivative. Water moves into the vacuole of the cell, increasing its lumen area, until the final size is reached. When the lumen reaches its final size, cells begin to mature, through the deposition of cell wall and lignification. When cell wall deposition ends, the protoplast autolysis and xylogenesis is complete.

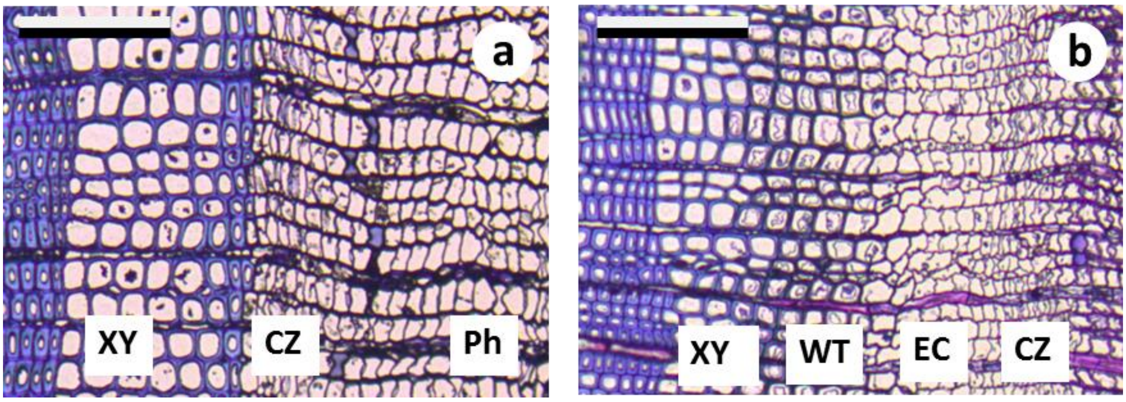

Figure 1.2 Transversal sections of dormant (a) and active (b) cambium of maritime pine (*Pinus pinaster*). CZ cambial zone; XY mature xylem; PH phloem; EC enlarging cells; WT cell wall thickening cells. Scale bar 250 µm.

1.4 The wood: secondary xylem

Secondary xylem is a conductive tissue, providing both mechanical strength and long-distance transport of water and nutrients, which enables shoots of some woody plants to grow up to 100 m tall (Taiz and Zeiger 2006). There are two types of wood: hardwoods and softwood (Figure 1.3). Hardwoods are found on Angiosperms and softwoods on Gymnosperms. The major difference between these two types of woods is the conductive elements. The main conducting elements in angiosperms are vessel elements, and in Gymnosperms tracheids (Figure 1.3). Both are elongated cells with secondary walls that lack protoplast at maturity. Tracheids are fusiform-shaped cells arranged in overlapping vertical files connected through pits. Unlike tracheids, vessel elements contain perforations, which are holes in the secondary walls that allow the communication between vessel elements. Perforations generally occur on the end of the walls, with the vessel elements joined end-to-end, forming long, continuous columns called vessels.

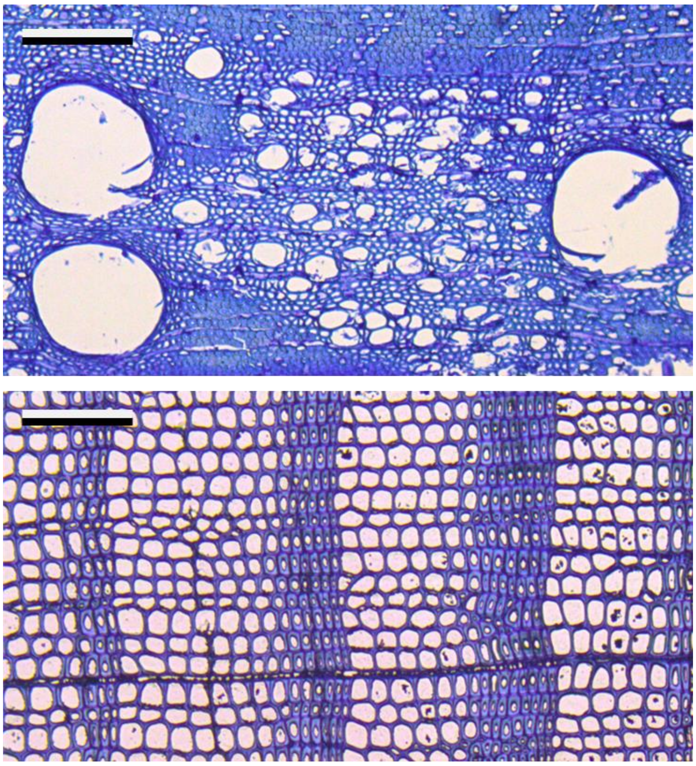

Figure 1.3 Transversal section of hardwood (top picture; *Quercus faginea*) and softwood (bottom picture; *Pinus pinaster*). Scale bar 250 µm.

1.5 Objectives

Although a strong relation between tree-ring width and climate has been established, dendrochronological studies are performed retrospectively using correlations between chronologies of tree-ring width and monthly climatic conditions, without considering wood formation at the intra-annual time scale. Since cambial activity changes throughout the growing season, the study of its dynamic and wood formation at an intra-annual time scale would provide a better understanding of the process and how it is controlled by climatic factors. Intra-annual analysis of wood formation decomposes the growing season into shorter periods of time by sampling in narrower time intervals (from minutes to weeks). This approach provides chronologies of the developing cell numbers or stem radial variation, which allow the study of cambial activity and wood formation throughout the year.

The seasonal dynamics of cambial activity and wood formation have been widely studied in temperate and boreal climates, however cambial activity in drought-prone environments, such as the Mediterranean, still remains poorly understood. The main objective of this dissertation is to study the daily and seasonal dynamics of cambial activity and wood formation in maritime pine growing under Mediterranean climate. In order to do so, the following specific objectives were established:

1. Determine the influence of timings and rates of cell production on xylogenesis;
2. Establish what causes the differences in diameter of trees with the same age;
3. Ascertain the influence of climate on cambial activity and xylem differentiation;
4. Define the daily and seasonal dynamics of stem radial variation and its relation to water availability;
5. And determine whether the bimodal pattern of growth, typical of the Mediterranean region, originates from a double reactivation of the cambium.

The objectives were pursued in four chapters, each one focusing on different aspects of the cambial activity of maritime pine:

In **Chapter II**, the timings and duration of xylem formation in trees of the same age and size but different growth rates are described. The aim of this chapter is to determine whether the differences in tree-ring width result from different cell production rates and if the rate of cell production influences the timings of xylogenesis. It is also studied whether the bimodal stem radial increment pattern, typical of the Mediterranean, originates from a double reactivation of the cambium.

In **Chapter III** the wood formation of trees with different sizes but similar age is described in order to determine if the differences in tree size are caused by different timings of cambial activity or by different rates of cell production.

In **Chapter IV**, cambial activity and xylem differentiation of maritime pine trees are monitored over two years in order to investigate the influence of climate on the timings of cambial activity.

In **Chapter V**, the hourly variation in stem radial increment and the water status of maritime pine are investigated using automatic dendrometers. The main objective is to define the daily and seasonal patterns of stem radius.

Finally, the last chapter (**Chapter VI**) resumes the main findings of this dissertation and presents future perspectives.

CHAPTER II

Are neighboring trees in tune? – Wood formation in *Pinus pinaster*

2.1 Introduction

The period in which wood formation occurs is the time window when environmental factors can act directly on the cells forming the tree ring and, consequently, on wood characteristics and properties. Thus, understanding the mechanisms of xylem development, namely the environmental factors responsible for the activation and cessation of radial growth, can have a great ecological and economic importance.

Xylem growth is a complex mechanism involving increases in the number of cells produced by the division of initials in the cambial zone, and in the volume of derivatives, which undergo differentiation before their complete maturation and functionality. Recently, there have been major steps forward in understanding cambial activity and wood formation (Deslauriers *et al.* 2009; Fonti *et al.* 2010; Gruber *et al.* 2010). In temperate and cold environments, there are evidences that temperature is a key factor for xylem growth, mainly in spring and autumn, as observed in the field (Vaganov *et al.* 2005; Rossi *et al.* 2007; Begum *et al.* 2008; Deslauriers *et al.* 2008; Seo *et al.* 2008) or with manipulation experiments (Oribe *et al.* 2001; Gričar *et al.* 2006; Begum *et al.* 2007). Nonetheless, trees living in the same site and subjected to the same climate can exhibit different growing dynamics and periods of growth (Rathgeber *et al.* 2011). This clearly indicates that factors other than climate can play a role in determining the timings of xylem formation.

In conifers of the Alpine timberline, xylem phenology is not constant throughout the tree’s lifespan, with older trees showing shorter and delayed periods of cambial activity and xylem cell differentiation than adult trees (Rossi *et al.* 2008). However, the older trees considered in that study were also taller and larger, thus the effect of age was not definitively disentangled from tree size. The shorter durations of xylogenesis observed in older trees by Rossi *et al.* (2008) could then be related to the size effect and not to age *per se*. In another study Rathgeber *et al.* (2011) removed the age factor by investigating xylogenesis in an even-aged plantation of silver fir. Trees showed the same age and similar heights, but belonged to different social classes. Cambial activity started earlier, stopped later, lasted longer and was more intense in dominant individuals than in intermediate and suppressed ones. Moreover, variability in tree-ring width was mostly explained by the rate of cell production, and only partially by the duration of growth. Since dominant trees were those with larger stem diameters and greater annual radial increments, it was unclear if either or both factors were a significant component of the growth process. Thus, the question whether cambial phenology is age and/or size related remains unraveled.

The Mediterranean climate is characterized by dry and hot summers and wet and cold winters, both periods unfavorable for growth (Mitrakos 1980; Terradas and Save 1992; Larcher 2000). Trees from these environments have physiological and morphological features (Cherubini *et al.* 2003; Battipaglia *et al.* 2010) as well as phenological adaptations (Llorens *et al.* 2003; Gratani *et al.* 2008; Montserrat-Marti *et al.* 2009) to survive in this double-stressed climate. Thus, two physiologically active periods are observed, with photosynthesis, shoot elongation and leaf flushing occurring during the warm and rainy months of spring and autumn. Similarly, adaptations to the Mediterranean climate have been observed in the timings of cambial activity, with the presence of bimodal patterns of xylem growth (Linares *et al.* 2009; Battipaglia *et al.* 2010; Camarero *et al.* 2010; de Luis *et al.* 2011; Gutierrez *et al.* 2011). The two periods of growth in spring and autumn are separated by a drought-imposed quiescent period in summer (Larcher 2000; Cherubini *et al.* 2003). Is the bimodal growth pattern observed in Mediterranean areas generated by an autumnal reactivation of cambium or by a swelling of previously produced cells or by both components? Either process can be monitored with anatomical observations of xylem development or dendrometers, respectively, but to our knowledge, the two techniques are rarely used together (Deslauriers *et al.* 2007; Makinën *et al.* 2008; Camarero *et al.* 2010). The present study investigated timings and duration of xylem formation of maritime pine (*Pinus pinaster* Ait.) using both anatomical observations and band dendrometers. Trees with the same age and size but different annual growth rates in the last 15 years (1994-2009) were selected in a coastal stand in Portugal with the aim of testing the hypotheses that 1) the differences in tree-ring width are a result of cell production and that cell production affects timings of xylogenesis and 2) the bimodal pattern originates from a double reactivation of cambial activity in spring and autumn.

2.2 Material and Methods

2.2.1 Study site: Perimetro forestal dunas de Cantanhede

The studies described in the following chapters were all carried out in Perimetro Florestal Dunas de Cantenhede (40°21’35.15’’ N, 8°49’10.06’’ W), located in the west coast of Portugal (Figure 2.1). It is a naturally regenerated plantation of maritime pine (*Pinus pinaster* Ait.). The management of the plantation consists mainly in a selective thinning performed 25-30 years after the last final cut. The selective thinning removes all of trees with a diameter at breast height inferior to 10 cm, leaving only the dominant and co-dominant specimens (Amaral 1980).

The selected forest stand presented trees of approximately 50 ± 5 years and a density of c.a. 230 trees per hectare.

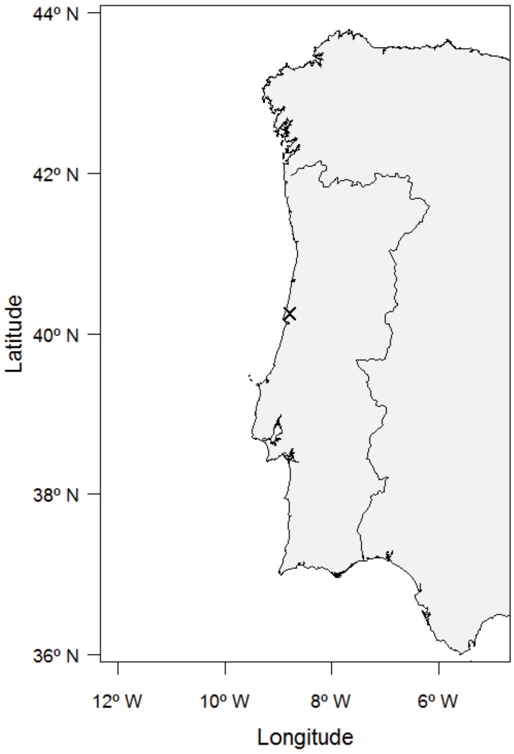

Figure 2.1 Map of Portugal with the study site location, Perimetro Florestal Dunas de Cantanhede, marked with a cross (×).

Although it is a monospecific plantation, the shrub layer contains a high variety of species, such as: *Calluna vulgaris* (L.) Hull, *Chamaespartium tridentatum* L., *Cistus crispus* L., *Cistus ladanifer* L., *Corema album* (L.) D. Don., *Cytisus grandiflorus* (Brot) DC.*, Cytisus scoparius* (L.) Link, *Erica arborea* L., *Halimium halimifolium* L., *Lavandula stoechas* L., *Rosmarinus officinalis* L., *Ulex* sp, and the invasive species *Acacia longifolia* (Andrews) Willd (Ferreira *et al.* 2010). The soil type is podzol, developed from sand and sandstone. It has a coarse granulometry and sandy texture in all horizons, which gives it a low water holding capacity (Ferreira *et al.* 2010). The pH is of 6.5 and the organic matter content of 0.17% (Srecu 2011).

The climate is typically Mediterranean with a strong oceanic influence. Precipitation occurs mainly in autumn and winter and the summer is characterized by a pronounced drought (Figure 2.2). The long-term (1950-2011) mean annual temperature is 16.1 ^{o}C, and the total annual precipitation of 965 mm (CRU). Daily maximum and minimum temperature and total precipitation data were acquired from the nearest meteorological station (Instituto Português de Meteorologia), located in Figueira da Foz, at 25 km south from the study site.

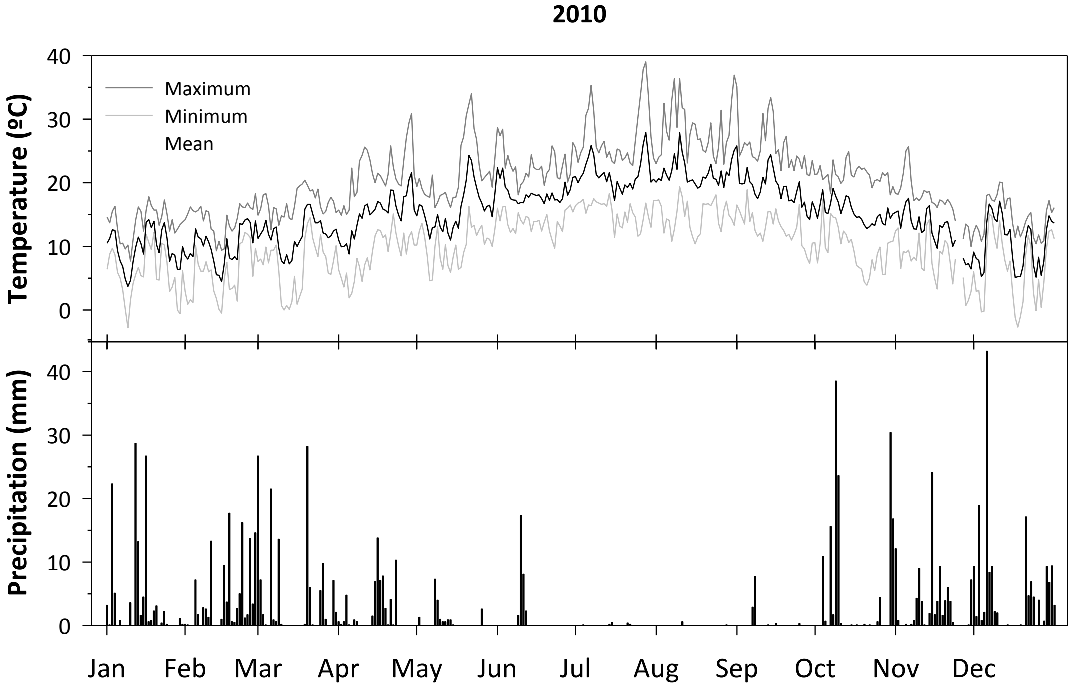

Figure 2.2 Daily temperature and precipitation in Figueira da Foz during 2010, at 25 km south from the study site (data from Instituto Nacional de Meteorologia, Portugal).

2.2.2 Maritime pine (Pinus pinaster Ait.*)*

The studies presented in this dissertation were performed on maritime pine (*Pinus pinaster* Ait.). Maritime pine is a typical Mediterranean conifer that represents 31 % of the Portuguese forest (Godinho-Ferreira *et al.* 2005). Although there are other species representative of the Portuguese forest, like *Quercus ilex* L. and *Quercus suber* L., conifers are model species for anatomical studies on wood formation. The homogeneous xylem with just one type of cells (tracheids) and the softwood make conifers perfect for wood anatomy studies, like the ones developed in this dissertation.

The distribution of maritime pine covers the Mediterranean basin and North Africa (Pereira 2002). In Portugal, is mostly distributed in the West coast. Historically, maritime pine was planted to prevent the advance of sand dunes to cultivated areas. Apart from dune stabilization, maritime pine is also an important source of wood and resin. Although there is some contradiction on whether or not maritime pine occurs naturally in Portugal or if it was introduced, palynological records have confirmed the presence of these species since Holocene (Morales-Molino *et al.* 2012). Maritime pine grows on low fertility soils under a wide range of water availability, from humid oceanic in France to arid conditions in central Spain and North Africa (Loustau *et al.* 1996). Maritime pine is also a shade-intolerant species, which is in agreement with its early succession stage (Zavala and Zea 2004).

Figure 2.3 Maritime pine (*Pinus pinaster* Ait.) at the study site, Perimetro Florestal Dunas de Cantanhede.

2.2.3 Tree selection

The selected forest stand had a density of approximately 230 trees per hectare (Figure 2.3) and trees presented similar characteristics in terms of dominance, height and vigor (Table 2.1). All trees were dominant or co-dominant with an average cambial age of 45 years at breast height (Table 2.1). In December of 2009, 35 trees were sampled with an increment borer. A core was taken at breast height in the south direction. The cores were air dried and sanded until the tree-ring patterns were perfectly visible. Tree-ring width was measured to the nearest 0.01mm using a linear table, LINTAB (Frank Rinn S.A, Heidelberg, Germany) and the program TSAP-Win (Rinn 2003). Based on the tree-ring widths from the previous 15 years (1994-2009), the trees were divided in fast- (F-trees) and slow-growing trees (S-trees) (Figure 2.4). From the initial 35 trees, 10 were selected for monitoring cambial activity and 25 for diameter variation. At the end of the monitoring season four trees were excluded from the analysis because no growth was observed in 2010. Thus, 8 trees (4 F- and 4 S-trees) were analyzed for cambial activity and 22 trees (12 F- and 11 S-trees) were monitored for diameter variation.

For each tree, height and crown diameter were measured in 2009. Diameter at breast height (DBH) was measured in the wood cores, from the last ring to the pith. Tree height was measured with an electronic clinometer and crown diameter calculated as twice the quadratic mean of the four cardinal radii of the projected crown.

Table 2.1 Diameter at breast height (DBH), height and crown diameter of fast- (F-trees) and slow-growing trees (S-trees) (mean ± standard deviation).

	Xylogenesis		Dendrometers	
Class	F-Trees	S-Trees	F-Trees	S-Trees
DBH (cm)	12.97 ± 1.15	12.05 ± 1.28	23.58 ± 4.77	24.64 ± 3.09
Height (m)	15.42 ± 0.92	15.02 ± 0.32	15.18 ± 0.85	15.45 ± 0.68
Crown diameter (m^2)	10.68 ± 0.26	9.82 ± 1.33	10.74 ± 1.46	7.62 ± 1.66

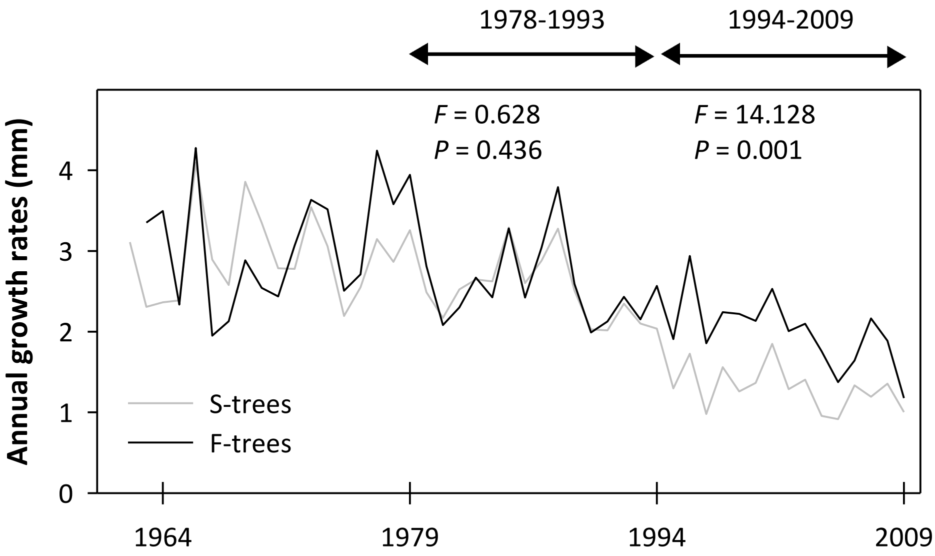

Figure 2.4 Annual time series of slow- (S-trees) and fast-growing trees (F-trees) used for anatomical investigation and dendrometer measurements in 2010. *F*-values are the values of the two-way ANOVA analysis of variance in each 15-year window period (1978-1993: synchronized period / 1994-2009: different growth-rate period).

2.2.2 Xylem formation

Sampling was performed from March to December 2010, [62-349 Day Of the Year (DOY)] by weekly collecting microcores on the stem using a Trephor (Rossi *et al.* 2006). The microcores were collected from 30 cm below and above breast height, in a spiral pattern on the south-facing side of the tree stem. Before sampling, bark was removed in order to reach the living tissues. Between two successive sampling dates, microcores were collected at least 5 cm apart to prevent getting resin ducts from adjacent sampling points. The microcores were placed in eppendorfs filled with alcohol (50 % in water) and stored at 5 ºC to avoid tissue deterioration. In the laboratory, the microcores were dehydrated through successive immersions in alcohol and D-limonene and embedded in paraffin (Rossi *et al.* 2006). Transverse sections 6-10 µm thick were cut from the samples with a rotary microtome, stained with cresyl violet acetate (0.16% in water), and immediately observed in a microscope (400-500 x magnification), under visible and polarized light to distinguish the developing xylem cells. Cambial and enlarging cells only have primary cell walls, which, unlike secondary walls, do not shine under polarized light (Figure 2.5). Cambial cells are characterized by thin cell walls and small radial diameters, while enlarging cells have a diameter at least twice that of a cambial cell (Figure 2.5-b). Wall thickening cells shine under polarized light and show a light violet coloration changing to dark violet at the end of maturation (Figure 2.5-c). Lignification appears as a color change from violet to blue, starting at the cell corners and middle lamella and spreading centripetally into the secondary walls. When the entire cell wall presents a blue coloration, lignification is complete and tracheids reach maturity (Gričar *et al.* 2005). In each sample, the number of cambial and developing cells was counted along three radial rows and averaged per class. Xylem formation was considered to have begun when an average of at least one enlarging cell was observed in each class. Xylem differentiation was considered complete when no cells were observed in wall thickening phase.

2.2.3 Band dendrometers

To estimate the changes in stem diameter, band dendrometers made of astralon (model D1-L, UMS, Munich, Germany) were installed on the stem of 25 trees at breast height. Installation was performed in January 2010, which allowed for a period of adjustment before the beginning of the growing season (Linares *et al.* 2010). Before the installation of dendrometers, the bark was carefully removed with a chisel to adjust the band dendrometer to the stem and to reduce as much as possible non-xylematic sources of swelling and shrinking (Zweifel *et al.* 2006). Band dendrometers were read weekly to the nearest 0.1mm. To avoid biases due to the circadian rhythms of water storage and depletion, all measurements were done in early morning (Linares *et al.* 2009).

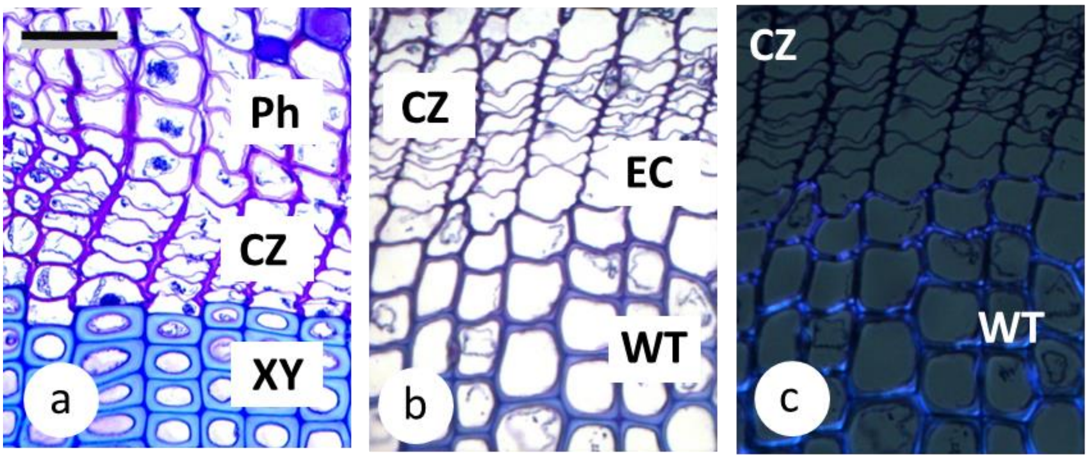

Figure 2.5 Transverse sections of the cambial zone under visible (a and b) and polarized light (c). DC: dormant cambium; XY: xylem; PH: phloem; EC: enlarging cells; WT wall thickening cells. Scale bar = 50 µm.

2.2.4 Statistical analysis

The characteristics of the F- and S-trees (diameter, height and crown diameter) selected for the xylogenesis and dendrometer analyses were compared using a two-way analysis of variance (ANOVA). Tree-ring width was divided in two 15-year periods and compared using a two-way ANOVA, to test for differences between the S- and F- trees and between both periods. Mean cell distribution was compared between F-and S-trees using the non-parametric Kruskal-Wallis test (χ^2). Cumulative and increment data from dendrometers were compared throughout the growing season using a PROC MULTTEST of the software package SAS version 9.2 (SAS Institute, Cary, NC). When performing many hypothesis tests on the same data set, this procedure excludes the probability of declaring false significances by adjusting the *P*-values using 10,000 bootstrap resamplings with replacement.

2.3 Results

2.3.1 Temperature and Precipitation in 2010

The minimum temperature in 2010 fell below zero in only three occasions, on January 9th and 29th and on February 14th (Figure 2.1). Mean daily temperature in January and February ranged between 3 and 13 ºC, with an average minimum temperature of 8 ºC measured during the week prior to the beginning of sampling. In spring and summer, temperature raised reaching a maximum of 39 ºC in mid-July. In September, temperature started gradually to decrease, but mean temperatures were still above 15 ºC. Temperature only dropped below 10 ºC in mid-November. Precipitation was more frequent in January-March and October-November. In April, precipitation started to decrease and very few precipitation events were observed from June to September, with a total of 43 mm of rain recorded during that period. The first precipitation event in autumn occurred at the beginning of October, with a total of 90 mm of rain in one week.

2.3.2 Tree characteristics

Tree-ring width measurements showed that in the last 15 years F and S-trees were significantly different (F = 14.128; P = 0.001) despite the fact that in the previous period (1978-1993) this difference was not observed (F = 0.628; P = 0.436; Figure 2.4). DBH ranged from 20 to 27 cm, tree height between 15 and 16.5 m and crown diameter between 7.62 and 10.74 m^2 (Table 2.1). No significant statistical differences were observed between F and S-trees concerning tree height (F = 0.0157; P = 0.901), DBH (F = 0.333; P = 0.569) and crown diameter (F = 3.036; P = 0.093).

2.3.3 Xylem phenology

The overall pattern of the differentiating tracheids showed a clear variation during the year in terms of number of cells (Figure 2.6). Cells in cambium, enlargement and cell wall thickening followed the typical bell shaped distribution, although there were still cells in wall thickening when the sampling stopped (DOY 349). Mature cells exhibited a growth curve with a plateau achieved at the end of summer. The number of cells in F-trees was always higher than in S-trees for all differentiation phases, except for cambium in spring. Statistics confirmed these observations, with the frequency distribution of the number of cells in enlargement (χ^2 = 6.97, $P < 0.01$), cell wall thickening (χ^2 = 23.03, $P < 0.001$) and mature cells (χ^2= 10.39, $P < 0.001$) being significantly different between the two groups of trees.

Winter samples revealed that there were 5-7 cells in the dormant cambium (Figure 2.6). The first samples taken in March (DOY 62), had 6-9 and 5-7 cambial cells in F- and S-trees respectively, which suggests that the cambium was already active. The maximum number of cambium cells occurred in June, followed by a slow decrease until October. The first tangential row of enlarging cells was observed in F-trees on DOY 76 (mid-March), and a week later in S-

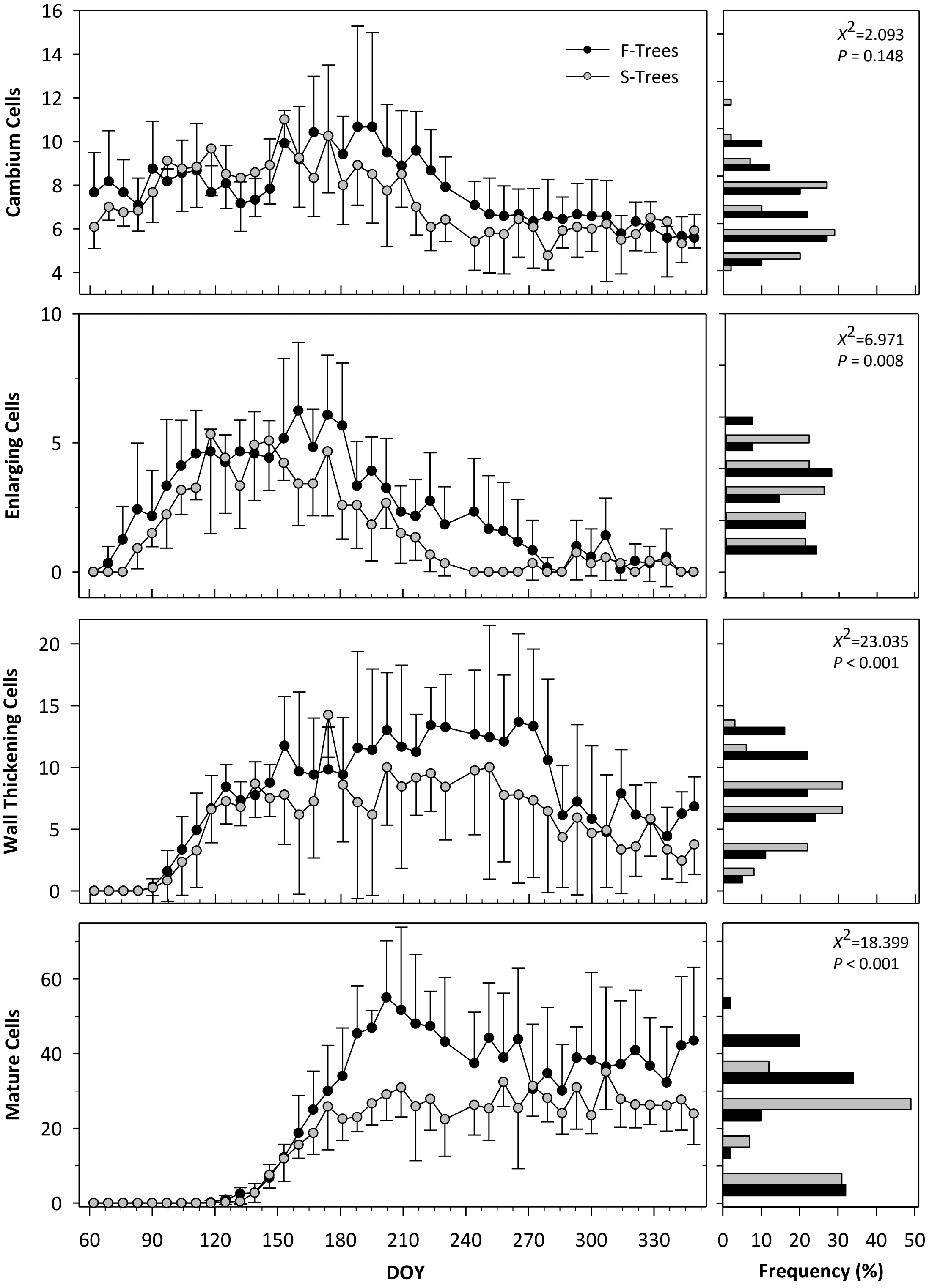

Figure 2.6 Number and frequency of cambial, enlarging, cell wall thickening and mature cells in fast- (F-trees) and slow-growing trees (S-trees). Vertical bars represent standard deviation; χ^2: Kruskal-Wallis test between F- and S-trees.

trees. The enlargement phase lasted until September (DOY 223) in S-trees while enlarging cells were still observed in October in F-trees (DOY 279). The last enlarging cells were observed in December (DOY 336) in F- and S-trees. Cell wall thickening and lignification was observed in April, 21 days after the beginning of enlargement, and started first in F-trees on DOY 97 (Figure 2.6). Since mid-July, S-trees exhibited a gradual reduction in the number of cells in wall thickening and lignification, while this reduction was observed in F-trees only in mid-October. The first mature cells were observed in May, on DOY 125, in F-trees, and two weeks later in S-trees. In December, the last sampling month, cells under lignification were still observed in both groups of trees, and the duration of the maturation phase could not be determined. At the end of sampling, F-trees produced 40 cells, about 15 more than those produced by S-trees.

2.3.4 Dynamics of diameter increment (Dendrometers)

Trees of both groups showed annual stem diameter increments with a clear bimodal pattern, characterized by a pronounced first period of increment in spring, a decrease in summer, and a second less marked period of increment in autumn (Figure 2.7, top graphics). Stem diameter increment started in mid-March (DOY 83) for both groups, but F-trees presented higher increment rates than S-trees. The maximum increment was reached at the beginning of May, followed by a decreasing trend, and a stem variation close to zero in August-September. The second period of positive increment was observed in October-December and corresponded with the first rainy event of autumn (compare Figure 2.7 with Figure 2.2). Several false significant probability scores were calculated for both weekly and cumulative increments (Figure 2.7, lower graphics). The high variation in stem diameter increment among trees hid all differences between the two groups of trees, and bootstrapped differences were only significant for the measurements performed at the end of May, on DOY 146 (Figure 2.7-A). More marked differences were detected using cumulative data (Figure 2.7-B), with patterns of F- and S-trees diverging since mid-April, when the bootstrapped adjusted P-values became significant ($P < 0.05$).

2.4 Discussion

The present study investigated timings and duration of cambium phenology and dynamics of xylem growth in fast and slow trees, growing in a Mediterranean climate with oceanic influence. Trees had the same age, similar height and crown diameter. Despite the differences in annual growth rates in the last 15 years, trees had similar DBH. Earlier onsets of cell enlargement in spring and the presence of a higher number of cells in cell wall thickening in winter were observed in F-trees. Also, since mid-April F-trees showed faster growth rates than S-trees, accumulating a higher number of cells in differentiation and mature xylem, confirming the hypothesis that cell production affects timings of xylogenesis. Although there was a clear bimodal pattern with two periods of stem increment in spring and autumn, no marked increase in the number of cambial cells was observed in autumn, suggesting that the second increment peak involved mainly changes in stem size. Consequently, the hypothesis that the bimodal growth pattern originates from a double period of cambial activity was not supported by the current findings.

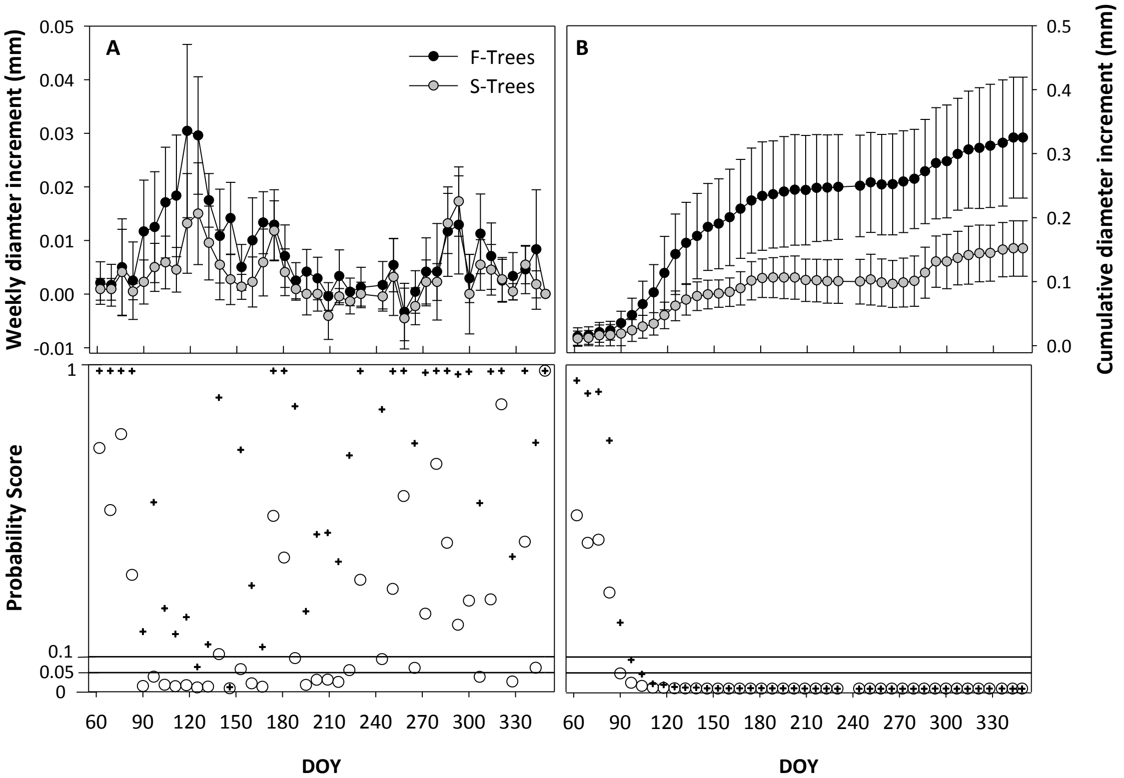

Figure 2.7 Weekly (A) and cumulative (B) increments by band dendrometers and probability scores comparing fast- (F-trees) and slow-growing trees (S-trees). Circles and crosses represent normal and adjusted p-values calculated by 10.000 bootstrap iterations with replacement, respectively.

Differences in xylem growth have already been described in trees of different age, size and vitality, and growing in different climatic conditions (Rossi *et al.* 2008; Gričar *et al.* 2009; Linares *et al.* 2009; Lupi *et al.* 2010; Rathgeber *et al.* 2011). However, the differences in the growing season observed in these studies could result from a number of different factors, such as size, age, or dominance (Rossi *et al.* 2008). Also, the comparison of wood formation in even-aged trees by Rathgeber *et al.* (2011) was not completely conclusive since trees belonged to different social classes and had different sizes. For the first time, an experimental design maintaining constant age and size was applied in trees growing in the same forest stand. The observed differences between F- and S-trees in the period of xylogenesis showed that cell production, and not age or size, played an important role determining timings of xylem phenology. Rossi et al. (2012) demonstrated that cell production is closely interconnected with the phenological phases of xylem according to a complex causal link. The date of onset and the rate of cambial division affect the number of cells produced by the cambium which, in turn, influences the ending of cell differentiation (Lupi *et al.* 2010; Rossi *et al.* 2012). Although cambial activity started one week earlier in F-trees, the wider tree-ring formed in F-trees was caused by a higher rate of cell division, rather than a longer period of xylogenesis. The higher number of cells produced by the cambium of F-trees, lead to a larger accumulation of cells in the developing xylem, which prolonged the differentiation phase and delayed the end of wood formation.

The analysis of the annual radial growth showed that F- and S-trees consistently diverged after 1994. A severe drought occurred in 1995, which might have reduced the competitive ability of some trees, causing the different growth rates observed afterwards. During periods of low precipitation better-adapted trees can maintain higher growth rates and also recover faster (Metsaranta and Lieffers 2008; Martinez-Vilalta *et al.* 2012). F-trees could have recovered faster from the 1995 drought due to genetic, morphological (e.g. rooting depth), physiological and/or microclimatic differences. Further studies are necessary to understand why, after 1995, trees of the same age and size, showed different growth rates.

Trees presented a clear bimodal pattern of stem diameter increment with two peaks, in spring and autumn, and a quiescent period in summer, which is characteristic of the vegetation from the Mediterranean region (Camarero *et al.* 2010; Gutierrez *et al.* 2011). Trees respond to water stress by closing the stomata and reducing leaf transpiration, which causes a progressive decline of CO_2 uptake (Cochard *et al.* 2000). The change in CO_2 uptake alters the proportion of carbon allocated to the different metabolic processes, which results in a decreased availability of carbon for those processes with lower priorities, such as cambial activity and secondary growth, which slow down or even cease (Oribe *et al.* 2003). In an early state of water deficit, cell expansion is restricted by the decline in turgor pressure, but if water stress persists cambial cell division can also be affected (Abe *et al.* 2003). De Luis et al. (2011) showed that water availability after the summer drought was the determining factor to increase the number of cambial cells of *P. halepensis* saplings subjected to different irrigation regimes.

However, in our case, the autumnal growth period did not correspond to a second period of cell division in the cambium. The autumnal recovery occurred shortly after the first rains and consisted mainly in a swelling of the outermost tissues of the stem and in the achievement of the pressure potential required for enlarging the few remaining undifferentiated cells, more likely produced by cambium during early summer (Abe *et al.* 2003; Rossi *et al.* 2009).

The second increment period was more evident on stem diameter variation than on cell anatomy studies. Stem diameter increment consists of several components including cell accumulation, and swelling and shrinking of the outermost tissues of stem due to water absorption and thermal variations (Zweifel *et al.* 2006). In areas with long dry periods, swelling and shrinking of the xylem and phloem can cause drastic changes in stem dimensions that exceed growth (Forster *et al.* 2000). In our study, the association of dendrometers and anatomical observations showed that the second increment period was due to variations in tree water status rather that new cell production by the cambium. Also, a 3-week delay was observed between the beginning of xylem differentiation (corresponding to the date of appearance of the first enlarging cell) and diameter increment. The resolution of dendrometers is probably not suitable to record microscopic variations caused by the first increases in cell number (Gruber *et al.* 2009). Other studies which have also compared band dendrometers and cellular analysis showed that indirect measurements fail in assessing the start of growth in spring (Makinën *et al.* 2008; Linares *et al.* 2009; Camarero *et al.* 2010). Nonetheless, unlike anatomical observations, dendrometers are a non-destructive method of measuring stem diameter fluctuations and can provide suitable estimates of xylem growth, after the non-xylematic sources of cyclic rhythms of water storage and depletion are removed (Zweifel *et al.* 2006; Turcotte *et al.* 2011).

2.5 Conclusion

As far as we know, this is the first study where cambial phenology was monitored in trees with similar age and size and different tree-ring widths. The results showed that F- and S-trees exhibited different rates of cell production and durations of xylogenesis. F-trees presented a higher rate of cell production which led to a prolonged xylem differentiation and the formation of wider tree rings. Thus, we can also conclude that the observed differences in xylogenesis resulted from cell production rather than age or size. The divergence in the annual growth rates of these trees started after a severe drought, with F-trees showing a faster recovery rate than S-trees. Also, the combined analysis of anatomical observations and dendrometers demonstrated that the bimodal pattern of maritime pine in 2010 was related to changes in stem size rather than an autumnal resumption of cambial activity.

CHAPTER III

The effect of size on maritime pine cambial activity in the Mediterranean region

3.1 Introduction

Forests are dynamic and complex ecosystems, with several extrinsic (*e.g.* climate, competition) and intrinsic factors (*e.g.* age, plant hormones) conditioning tree growth. The interaction of these factors is responsible for the variability of tree growth patterns observed in a forest. Has trees get older and taller, physiological processes such as photosynthetic capacity and hydraulic conductivity change (McDowell *et al.* 2005; Martinez-Vilalta *et al.* 2007). A study comparing the below-ground hydraulic conductance of Scots pine's observed that it declined with increasing age/size (Martinez-Vilalta *et al.* 2007). Older/taller trees compensated for the decreased condunctance by resorting to water stored in the ellastic tissues of the stem. The physiological mechanisms associated with the trees dimension have repercurssions on the climatic signals recorded in the tree rings. For instance, Carrer and Urbinati (2004) found that, at the timberline, tree-rings of older/taller trees were more sensitive to climate, whereas the opposite was observed in Mediterranean pines (Vieira *et al.* 2009). These contradictory findings could be explained by the difficulty in disentangling the effect of age from size.

The effects of age and size are also difficult to separate in wood formation studies. A study comparing xylogenesis in trees of different age from the alpine timberline revealed that old trees (> 250 yr) had a shorter and delayed period of cambial activity than adult trees (< 80 yr) (Rossi *et al.* 2008). However those older trees were also larger, thus the effect of age was not completely disentangled from size. With the aim of isolating the effect of size on xylem formation, Rathgeber *et al.* (2011) studied trees of different size and social status but similar age, growing in a plantation in France. They determined that the differences in tree size were due to the higher rate of cell production observed in the dominant trees. In maritime pine it was also observed that the rate of cell production was the responsible for the different tree-ring widths observed in the last 15 years (*Chapter II*). However, xylogenesis timings are also linked with cell production (Lupi *et al.* 2010; Rossi *et al.* 2012), with higher cell production associated with longer periods of xylogenesis (Lupi *et al.* 2010) .

Most of the studies describing wood formation and comparing it in trees of different size/age were carried out in boreal or temperate environments, where temperature is the main factor limiting tree growth. However, few studies were performed in water limited environments, such as the Mediterranean region. Climate is changing worldwide, with a predicted increase in temperature and a decrease in precipitation for the Mediterranean region (IPCC 2007). These changes could have important implications for tree growth, with an increase of water stress and resource competition (Martinez-Vilalta *et al.* 2008; Linares *et al.* 2009; Gómez-Aparicio *et* *al.* 2011). Among all the species of the Mediterranean forest, pines are the most sensitive to competition, reaching reductions of potential growth close to 100% under intense competition (Gómez-Aparicio *et al.* 2011). The sensitivity to competition of pines increases at decreasing precipitation, especially in smaller trees (Gómez-Aparicio *et al.* 2011). Vieira *et al.* (2009) compared the climatic response of maritime pines of different age and size growing in the Mediterranean region and observed that younger and smaller trees responded to climatic conditions earlier in the growing season. This result suggested that younger/smaller trees started to grow earlier, which indicates that there could be differences in the timings of xylogenesis between the age/size groups. However, it was observed that the rate of cell production, not timings of xylogenesis, was responsible for the differences in tree-ring width (*Chapter II*). In order to determine whether the differences in stem diameter observed in an even-aged stand of maritime pine in Portugal are due to different rates of cell production or xylogenesis timings, the following hypotheses were tested: 1) growth onset occurs simultaneously in both diameter classes; 2) the rate of xylem cell production is higher in larger trees, and 3) the period of xylogenesis is longer in larger trees.

3.2 Material and Methods

3.2.1 Study site and tree selection

The study site is described in section *2.2.1.* The selected trees for this study had an average age at breast height of 55 years and were divided in two groups according to the frequency distribution of stem diameters determined by Campelo *et al.* (2013): large trees (L-trees; 38.7 ± 3.9 cm) and small trees (S-trees; 23.9 ± 3.0 cm). Five trees were selected from each diameter class to monitor cambial activity and stem diameter variation during 2011. Height, age and diameter at breast height (DBH) from the two diameter classes were compared using *t-tests* (Table 3.1).

Table 3.1 Average diameter at breast height (DBH), height and age of small (S) and large (L) trees (± standard deviation) and *t-test* comparing both diameter classes (n = 5)

Class	S-trees	L-trees	*t-test*	*P*
Diameter (cm)	22.5 ± 1.9	37.9 ± 2.6	10.58	< 0.001
Height (m)	14.8 ± 0.8	17.0 ± 1.2	3.41	0.009
Age (years)	45.6 ± 4.9	48.8 ± 4.4	1.08	0.312

3.2.2 Xylem development

Sampling was performed from March 2011 to February 2012 (62-409 Day Of Year [DOY]), by weekly collecting microcores on the stem using a Trephor (Rossi *et al.* 2006). The collection and processing of the microcores samplings was performed following the procedure described in section *2.2.2*.

3.2.3 Xylem phenology

The phenology of xylem development was compared between both diameter classes. For each tree, two phenophases were considered: cell enlargement and cell wall deposition. The beginning of each phase was defined as the date when more than 50 % of the observed radial paths showed at least one cell in that phase. The end of each phase was defined as the date when less than 50 % of the observed radial paths presented at most one cell in that phase. The duration of the phases was determined by the difference between onset and end of each phase. As the assumption of normality in data distribution was occasionally not fulfilled, differences in the timings and duration of the xylem phenology phases between diameter classes were tested using a Mann-Whitney rank sum test (*U*).

3.2.4 Cell measurements

When xylem formation was complete in February 2012, a microcore was collected from each tree, embedded in paraffin and processed following the procedure described in section *2.2.2*. The resulting sections were stained with 1% aqueous safranin and permanently fixed with Canada balsam (Eukitt). A camera fixed on an optical microscope was used for image analysis, at 20 × magnification. On each section, three radial paths were selected to measure lumen area and cell wall thickness, using Wincell (Regent Instruments Inc.). Tracheids were classified in earlywood and latewood following the Mork's formula described in Denne (1989). The measurements were averaged by diameter class and compared using a generalized linear mixed model in SAS 9.2 (SAS Institute Inc. Cary, North Carolina). Each of the parameters was individually tested for differences using a One-way ANOVA.

3.2.5 Band dendrometers

To estimate the changes in stem diameter, band dendrometers made of astralon (model D1-L, UMS, Munich, Germany) were installed on 25 trees at breast height. This was done in January 2011 to allow for a period of adjustment before the beginning of the growing season (Linares *et al.* 2010). Before the installation of dendrometers, the bark was carefully removed with a chisel to better adjust the band dendrometer to the stem and to reduce non-xylematic sources of swelling and shrinking as much as possible (Zweifel *et al.* 2006). Band dendrometers were read weekly to the nearest 0.01 mm. To avoid biases due to the circadian rhythms of water storage and depletion, all measurements were done in early morning (Linares *et al.* 2009).

3.3 Results

3.3.1 Temperature and precipitation in 2011

The average temperature in 2011 was 17.6 ^{o}C and total precipitation was 800 mm, 1.5 ^{o}C above and 165 mm below the long-term average, respectively (the long-term temperature and precipitation of the study site are described in section *2.2.1*). In January and February, the average minimum temperature was 5.3 ^{o}C, starting to rise in mid-March (DOY 75; Figure 3.1). From this point on, the average temperature was above 10 ^{o}C until mid-December (DOY 345). The average spring (March-May) and summer (June-August) temperatures were 16.0 ^{o}C and 19.4 ^{o}C, respectively. The temperature started to drop in October, and average temperatures below 10 ^{o}C were only observed in mid-December. Precipitation was more abundant in January-April 2011 and from October 2011 to February 2012. In May, precipitation decreased abruptly and very few events of precipitation were observed until September, with a total of only 73 mm of rain recorded during that period.

3.2.2 Xylem development and phenology

Dividing and differentiating tracheids showed clear variation patterns during the year in terms of number of cells (Figure 3.2). At the beginning of the sampling season, in March, the cambium of L-trees was already active, presenting the maximum number of cells observed (9 cells). S-trees achieved that number of cells one week later. The maximum number of cambial cells was observed between March and April in both groups. Afterwards, the number of cambial cells decreased progressively until reaching a minimum of five cells in the summer. In November the number of cambial cells increased again to 6-7 cells, remaining constant until the end of the study.

The first cells in enlargement were observed one week earlier in L-trees. However, the beginning of enlargement did not present statistical differences between the two diameter classes (U = 4.50; P = 0.190; Figure 3.3). The rate of cell production was higher in L-trees, which presented an average of five cells in enlargement for eight weeks (March-May), whereas S-trees presented a maximum of 4 cells only during two weeks at the end of March (Figure 3.2).

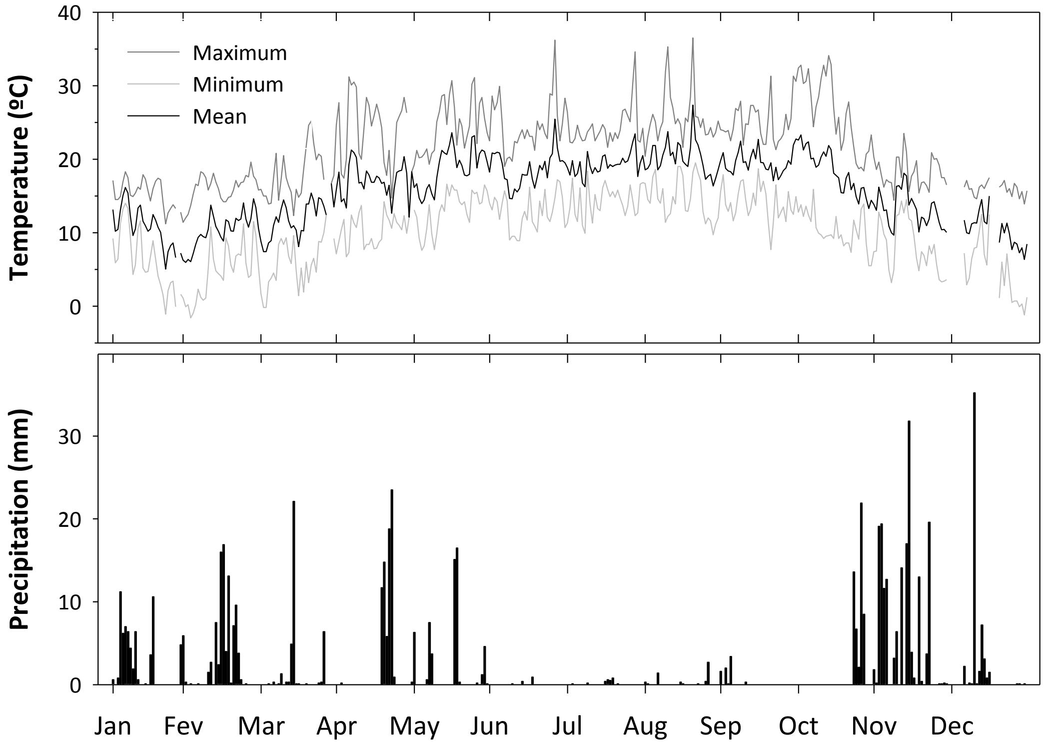

Figure 3.1 Daily values of temperature and precipitation in Figueira da Foz during 2011, at 25 km from the study site (ddata from Instituto Português de Meteorologia).

Overall, enlargement lasted longer in L-trees, which presented cells in this differentiation phase until mid-September, while the last enlarging cells were observed in S-trees at the end of June (*U* = 20; *P* = 0.016). The average duration of enlargement was 250 days in L-trees and 90 days in S-trees (*U* = 20; *P* = 0.016).

Cell wall deposition started one week earlier in L-trees, at the end of March. L-trees exhibited 6-7 cells in cell wall deposition for six months, from April to October. S-trees had a similar pattern, but with fewer cells (3-5) and during a shorter period, April to August (Figure 3.2). The beginning of cell wall deposition did not present significant differences in the two diameter classes (*U* = 3; *P* = 0.111). Cell wall deposition lasted until mid-September in the S-trees and until mi-November in L-trees (*U* = 20; *P* = 0.016). The duration of this phase lasted 250 days in the L-trees and 185 days in the S-trees (*U* = 20; *P* = 0.016). The first mature cells were observed simultaneously in both diameter classes in mid-April. At the end of the study, L-trees presented a total of 30 tracheids while S-trees only presented 15 tracheids.

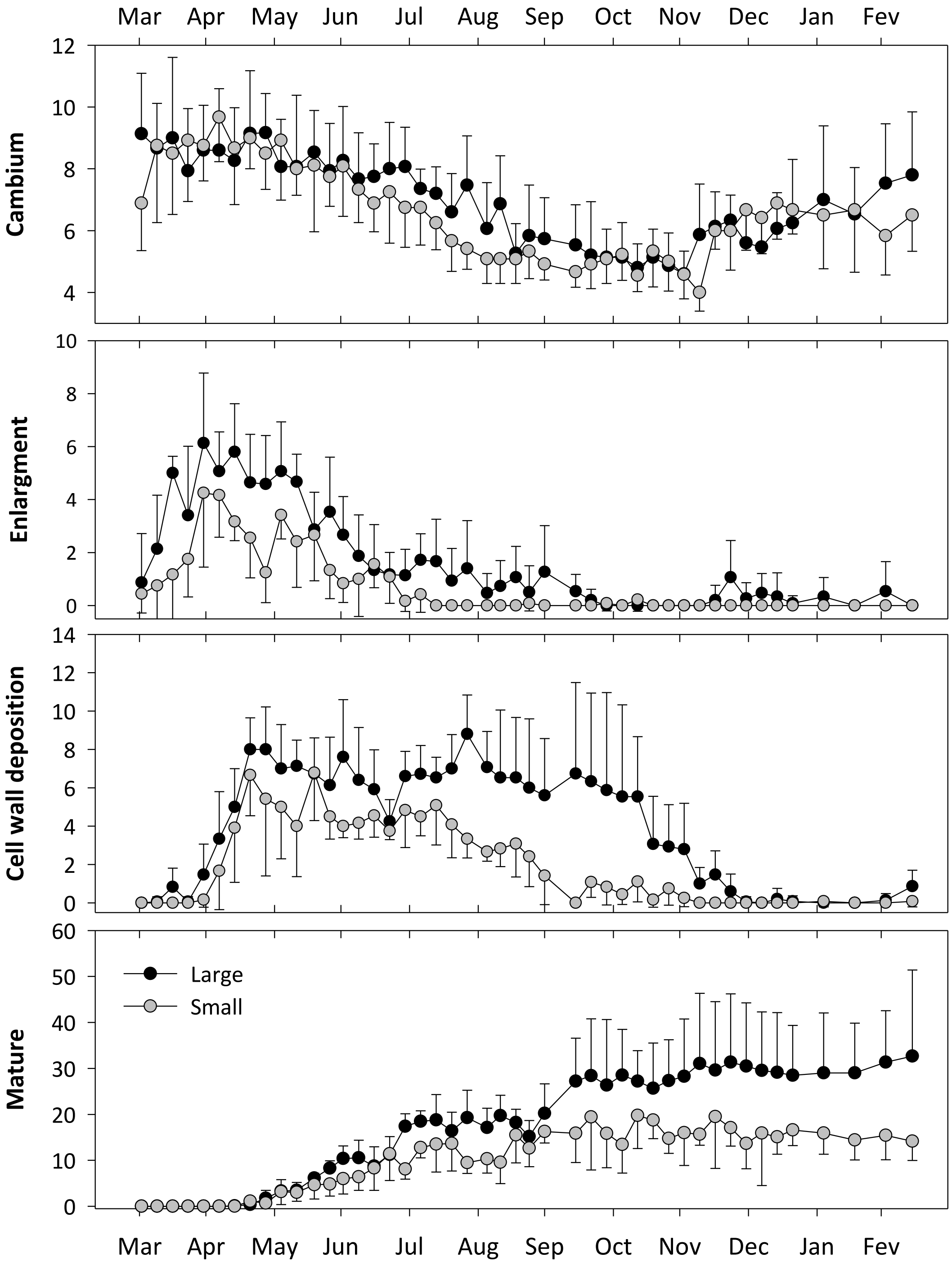

Figure 3.2 Number of cambial, enlargement, cell wall thickening and mature cells in Large and Small trees. Vertical bars represent standard deviation.

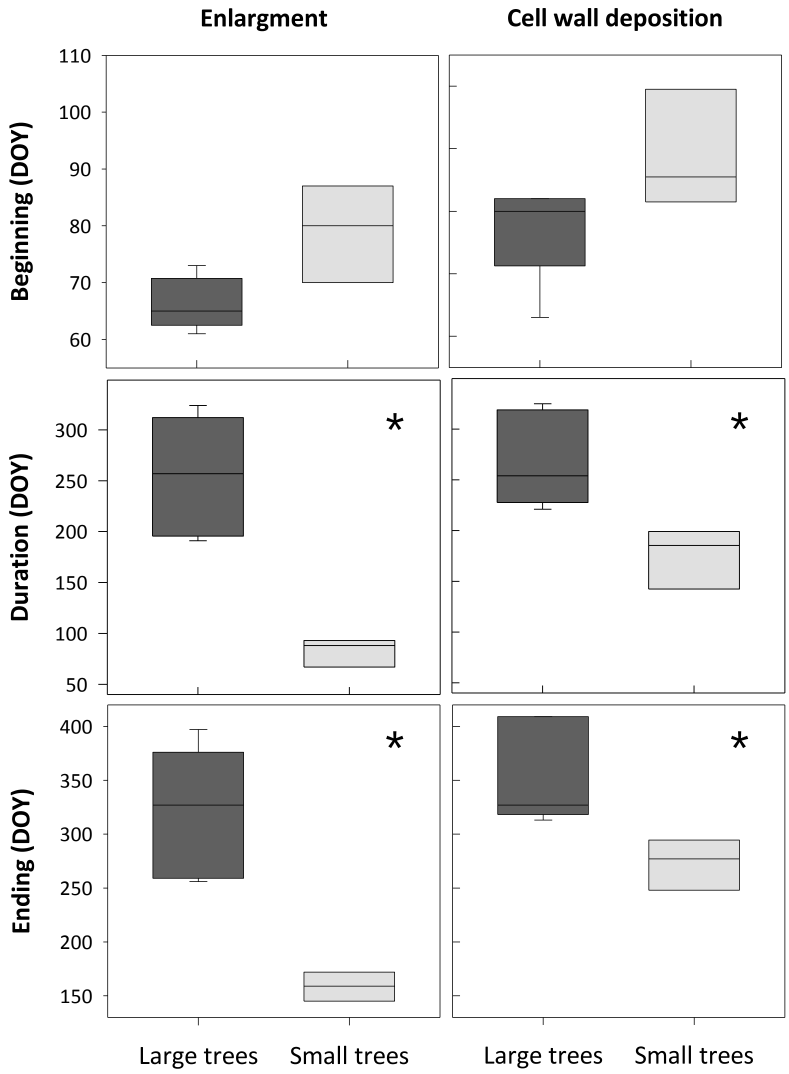

Figure 3.3 Beginning, duration and end of enlargement and cell wall thickening, in Large and Small trees. Horizontal line represents the median and the whiskers standard deviation; ** P < 0.05*.

3.3.3 Tracheids characteristics

When the tree ring was analyzed as a whole, the total number of tracheids (*F* = 2.56; *P* = 0.148), their lumen area (*F* = 2.45; *P* = 0.151) and cell wall thickness (*F* = 0.415; *P* = 0.538) did not present significant differences between L- and S-trees (Figure 3.4). However the proportion of earlywood (EW)/latewood (LW) revealed significant differences in the two diameter classes (*F* = 5.02; *P* = 0.036). L-trees presented an average of 17 EW tracheids in respect to 12 EW cells in S-trees (*F* = 1.201; *P* = 0.305), these tracheids presented a larger lumen area and thinner cell wall in L-trees than in S-trees, but the differences were not significant (*F* = 2.49; *P* = 0.149 and *F* = 0.10; *P* = 0.751, respectively). The number of LW tracheids was higher on L-trees (13) than on S-trees (4), however not significant (*F* = 3.722; *P* = 0.09). Although lumen area was slightly larger and cell wall thinner in L-trees, the differences were also not significant (*F* = 2.49; *P* = 0.130 and *F* = 0.24; *P* = 0.628, respectively).

3.3.4 Stem radius variations

In both classes, stem diameter increments showed a clear bimodal pattern, characterized by a pronounced increasing period in April, followed by a plateau in late spring and early summer, and a second less marked increase in autumn (Figure 3.5-A). The variation pattern showed a high synchrony between the two diameter classes. The maximum increment was observed in the beginning of April (DOY 100), with L-trees showing an increment of 2.8 x 10^{-2} mm and S-trees 2.0 x 10^{-2} mm. After the maximum, the increment started to decrease, reaching a variation close to zero in the period from August to mid-October. In mid-October a second increment period was observed in both classes with similar amplitudes (Figure 3.5-B). The total cumulative increment observed was higher in L-trees than in S-trees, with 33 x 10^{-2} and 19 x 10^{-2} mm, respectively.

3.4 Discussion

This study investigated timings of cambial reactivation and xylem differentiation in two diameter classes (large and small) from an even-aged maritime pine stand in Portugal. The cambium was active from March to July, and quiescent from August to November in both diameter classes. The annual periods of cambial activity observed in maritime pine suggested that, in the Mediterranean region, trees are under a double stress: low temperatures in the winter and high temperatures associated with low water availability in the summer. Enlargement started at the same time in both diameter classes, supporting the hypothesis that growth onset was not affected by tree size, which indicated that a common factor (e.g. temperature and/or photoperiod) was involved in the break of winter dormancy. The differences in the timings of xylogenesis between the two groups were observed in the end of enlargement and cell wall deposition, with both phases lasting longer in L-trees, confirming our third hypothesis. Apart from the different timings, the rate of cell production was also higher on L-trees.

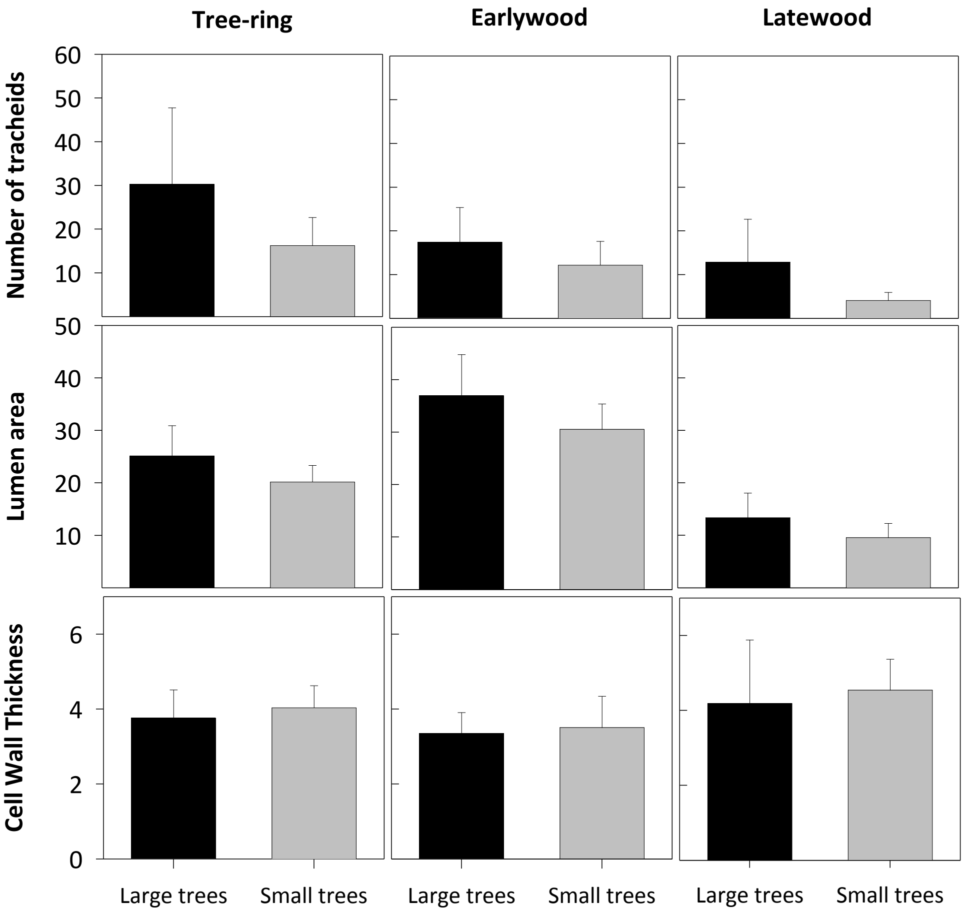

Figure 3.4 Number of tracheids, their lumen area and cell wall thickness in tree-ring, earlywood and latewood, for large and small trees.

3.4.1 Cambial dormancy and activity

Winter cambial dormancy in trees consists of two phases, the resting and the quiescent phase (Riding and Little 1984; Rensing and Samuels 2004). The resting phase corresponds to the period when the cambium is unable to produce new cells, even when supplied with auxin and under favorable conditions (Little and Bonga 1974). When the cambium regains the ability to produce new cells it is said to be quiescent (Little and Bonga 1974). Cambium is reactivated and quiescence broken when the environmental conditions are suitable for growth (Larson 1994). Thus, the cambial resting stage is determined by internal factors and the quiescent stage by external factors. The number of cambial cells in S-trees was the same in the start (March 2011) and in the end of the study (February 2012), suggesting that in March these trees were still in the quiescent stage. However, L-trees presented a higher number of cells in

March 2011 than in February 2012, suggesting that quiescence had been broken and that cambium was already active in the beginning of March.

The conversion from quiescent to active state is strongly connected with temperature (Korner 1998; Rossi *et al.* 2008; Begum *et al.* 2010). Rossi *et al.* (2008) determined that the minimum temperature threshold for tree growth in conifers from cold environments was between 4 and 5 °C. In localized heating experiments, the portion of the cambium heated started to divide earlier, demonstrating the importance of temperature in cambial reactivation (Oribe *et al.* 2001; Oribe *et al.* 2003; Gričar *et al.* 2006; Begum *et al.* 2007; Begum *et al.* 2010). In maritime pine, xylogenesis started earlier in years with a warmer winter (*Chapter IV*).

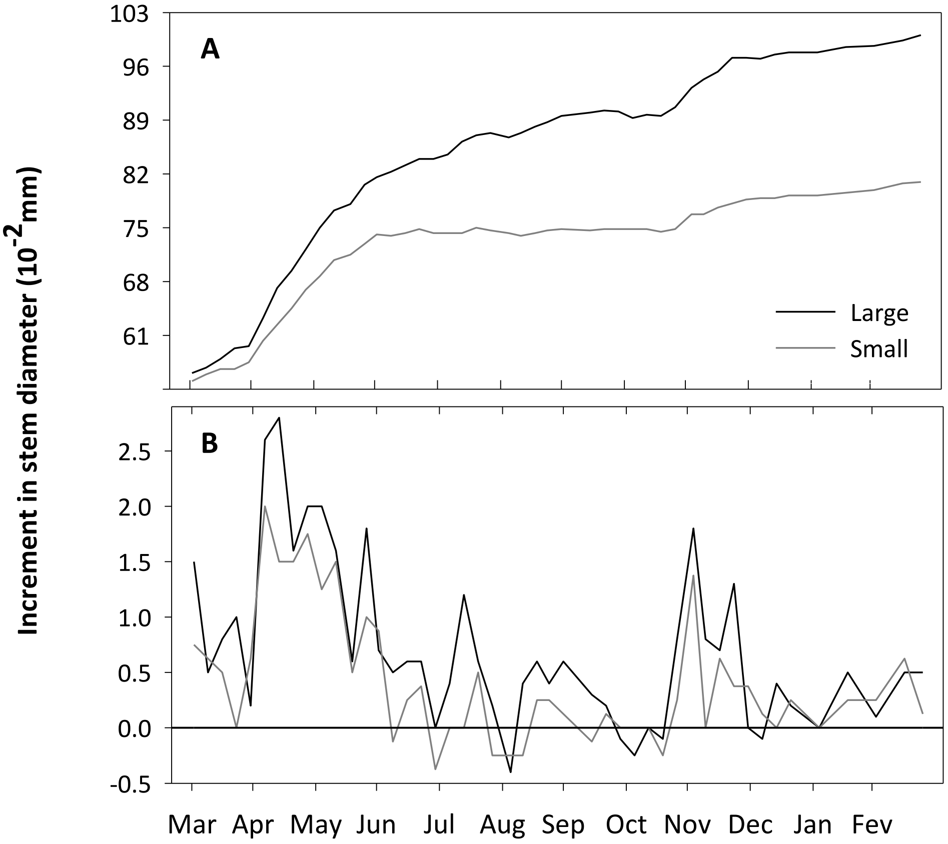

Figure 3.5 Average cumulative and weekly increments in stem diameter measured with band dendrometers in large and small trees.

In a literature review on the regulation of cambial activity in trees from temperate and cold environments, the resting phase was described to occur between November and December, the quiescent phase from January to March and cambial activity from April to October (Begum *et al.* (2013). The timing of cambial re-activation observed in maritime pine was in agreement with this classification, however the minimum number of cambial cells was observed in August-November, suggesting that the trees were quiescent during that period. The high temperatures registered during summer associated with water stress, can trigger trees to enter a quiescent state until the return of favorable environmental conditions (Sarris *et al.* 2013; *Chapter V*). The reduction of the number of cambial cells observed during the summer appears to be a defense mechanism from the harsh environmental conditions.

Summer quiescence was broken after the precipitation events that took place in late October. Precipitation re-hydrated the stem and brought the tree to a physiologically active state. The cambial activity observed in November indicates that maritime pine can resume growth after a period of drought (Vieira *et al.* 2009; Sarris *et al.* 2013). An increase in the number of cambial cells in October-November has also been observed in another Mediterranean species, *Juniperus thurifera*, however it was followed by the differentiation of new xylem cells (Camarero *et al.* 2010). Previous studies suggest that maritime pine also has the ability to form new xylem cells in autumn (Vieira *et al.* 2009; Campelo *et al.* 2013), however, the short photoperiod observed in November may have prevented it. The number of cambial cells remained constant from December to February, suggesting that the cambium was dormant. Further studies are necessary, namely heating experiments, in order to determine if there is true winter dormancy or if the cambium remains quiescent until spring. Studies in the cambial activity of Mediterranean trees have demonstrated that cambium can maintain activity during mild winters (Liphschitz and Levyadun 1986; Cherubini *et al.* 2003). Whatever the case may be, high temperatures and low water availability during summer were the main factors limiting cambial activity, and not winter temperatures.

The annual periods of cambial dormancy and activity in the Mediterranean region appear to be different from the ones proposed by Begum *et al.* (2013) and so should be the limiting factor(s). Tree growth in the Mediterranean region appears to be under a double stress: low temperatures and short photoperiod during winter and high temperatures, irradiance and low water availability in the summer. Although a clear transition between the different xylem differentiation phases was observed in 2011, the double stress and the year-to-year climatic variability typical of the Mediterranean region represent a new challenge for the general definition of cambial activity and dormancy.

3.4.2 Cambial activity and wood formation

Differences in the timings of cambial activity have been observed in trees of different age and social status (Rossi *et al.* 2008; Rathgeber *et al.* 2011). Cambial activity was reported to start earlier on younger (Rossi *et al.* 2008) and dominant trees (Rathgeber *et al.* 2011). Although, L-trees presented more cambial cells in the beginning of March and started to differentiate one week earlier than S-trees, this difference was not statistically significant. Contrary to the observations by Rathgeber *et al.* (2011), who found a strong relationship between the onset, duration and end of cambial activity and tree size, it was observed that only the duration and end of the xylem differentiation phases were related to tree size. L-trees were larger, taller and probably with better access to resources above- and belowground which allowed them to produce new xylem cells for a longer period of time. Additionally, L-trees showed higher rates of cell production in spring and, therefore, more time was needed to complete xylem maturation, leading to a later end of cambial activity (Lupi *et al.* 2010).

From the meteorological records, 2011 was an unusually hot and dry year (*Chapter IV*, Figure 4.1). In order to avoid drought-induced hydraulic failure, trees close their stomata (Ripullone *et al.* 2007; McDowell *et al.* 2008), limiting carbon uptake and eventually growth (Oribe *et al.* 2003; Michelot *et al.* 2012). Probably L-trees were able to prolong xylogenesis due to a deeper rooting system or to a higher amount of storage water (Phillips *et al.* 2003; Martinez-Vilalta *et al.* 2007). Phillips *et al.* (2003) showed that taller trees used a higher percentage of stored water to support daily water transport and that the use of stored water also increased photosynthesis on a daily basis. In fact, in maritime pine the number of cells in the wall deposition phase was kept nearly constant during the summer in L-trees, while S-trees showed a progressive reduction of the number of cells in that phase along the summer. This indirectly reveals that L-trees had more carbon resources.

Comparing the cellular parameters studied in the 2011 tree-ring (number, lumen area and cell wall thickness), between L- and S-trees, the only significant difference was the larger proportion of latewood in L-trees. Latewood is formed in response to a decline in water availability (Domec and Gartner 2002). Lower water contents decrease cell turgor resulting in smaller, denser and mechanically stronger tracheids (Abe *et al.* 2003; Sperry *et al.* 2006). Latewood may also provide water storage, with earlywood responsible for most of the water transport needs of a tree (Domec and Gartner 2002). The wider latewood present in L-trees can be explained by the better and prolonged access to water by those trees. A larger latewood is thus in agreement with the longer period of growth found in L-trees.

3.4.3 Diameter increment variations

The variation in diameter registered by the band dendrometers showed a clear synchrony between both diameter classes, although L-trees presented a higher increment. Diameter increment consists of several components, including the daily swelling and shrinking of the elastic tissues of the stem (Irvine and Grace 1997; Zweifel *et al.* 2006). The high synchrony observed between both diameter classes reflects the strong component of water in stem diameter increment. The onset and maximum stem diameter increments corresponded to the start and maximum number of cells observed in the enlargement phase. As observed in previous studies, the resolution of band dendrometers is probably not sufficient to record the microscopic variations caused by the first increases in cambial cell number (Gruber *et al.* 2009; *Chapter II*). The plateau and slight increment decrease observed in the summer corresponded to the stem de-hydration and contraction, triggered by high temperatures and low soil water availability (Zweifel *et al.* 2001; *Chapter V*). During the summer, trees are not able to completely compensate for the water lost during the day due to transpiration, contracting gradually from one cycle to the next (Devine and Harrington 2011; *Chapter V*). In autumn, precipitation triggered a second increment peak of stem diameter, corresponding to the re-hydration of the stem. Although the second peak also corresponded to the increase in the number of cambial cells observed in November, the main responsible for the second peak was stem re-hydration. In the end of the study, L-trees presented a bigger increment, which was in agreement with the higher number of cells observed in those trees.

3.5 Conclusions

For the first time, periods of cambial activity and dormancy were defined in trees with the same age and different size, growing under Mediterranean climate. Our findings supported the hypothesis that the cambial activity of maritime pine is adjusted to cope with the double climatic stress typical of the Mediterranean region, with low temperatures and reduced photoperiod in winter, and high temperature associated with low water availability in the summer. The simultaneous onset of the enlargement phase in both diameter classes indicates that the start of xylem differentiation was not mediated by size, leading us to admit that the start of cambial activity (or break of winter quiescent) should be triggered by the same factor (e.g. photoperiod and/or temperature). However, the longer duration of xylogenesis observed in L-trees was probably due to a better access to resources by those trees, allowing higher rates of cell production.

We propose that, in the Mediterranean region, although the break of winter dormancy is triggered by temperature, as in other environments (Temperate and Boreal), summer drought is equally limiting. Although significant improvements have been made in defining the annual periods of cambial activity and dormancy in the Mediterranean region, further studies are necessary in order to better describe the capacity of trees to adjust cambial activity to the current environmental conditions, namely, after the summer drought.

CHAPTER IV

Xylogenesis of Pinus pinaster under a Mediterranean climate

4.1 Introduction

Environmental factors affect wood formation at different levels, from a slight variation in lumen area to the complete absence of a tree-ring (Fritts 1976). In general, species of high latitudes and altitudes are mainly limited by temperature (Rossi *et al.* 2007), whereas temperate species are controlled by a combination of temperature and water availability (Carrer and Urbinati 2006). In dryer areas such as the Mediterranean region however, tree growth is mainly limited by water availability (Cherubini *et al.* 2003). Several dendrochronological studies carried out in this region have demonstrated the importance of water availability, especially during spring, on tree growth. A positive relation between earlywood widths and May precipitation was observed in maritime (*Pinus pinaster* Ait.) and Aleppo pines (*Pinus halepensis* L.), two typical Mediterranean species (de Luis *et al.* 2009; Vieira *et al.* 2010; Campelo *et al.* 2013). A broader study by Lebourgeois *et al.* (2012) concluded that spring drought (May-June) was the primary factor limiting conifer growth in the Mediterranean, with narrower tree rings being observed in years with a drier spring.

Although a strong relation between tree-ring width and climate has been established in the Mediterranean area, all of the above-mentioned studies were performed retrospectively, using correlations between tree-ring width series and monthly climatic variables, without considering wood formation at the intra-annual scale. Studies on the wood formation of Spanish juniper (*Juniperus thurifera* L.) and Aleppo pine have described xylem production to occur in two different seasonal periods: spring and fall (de Luis *et al.* 2007; Camarero *et al.* 2010). This growth pattern has been described as the bimodal growth pattern and is characteristic of water limited environments, such as the Mediterranean, the desert and tropical woody species (Liphschitz and Levyadun 1986; Camarero *et al.* 2010). Apart from the seasonal pattern of xylem growth, Camarero *et al.* (2010) also observed that onset and duration of xylogenesis were mainly determined by temperature. In maritime pine however, it was determined that xylogenesis was mainly determined by the rate of cell production, with trees with higher cell production presenting a longer growing period (*Chapter II*).

Even though there are some studies on the cambial activity of trees from water limited environments, the established knowledge is still fragmentary, especially in terms of the climatic influence on xylogenesis. A strong relation between temperature and cambial activity has been reported for conifers of colds environments, but the effect of water stress on drier climates still remains poorly understood. The climate is changing and the frequency of extreme warm years in Europe is expected to increase (IPCC 2007). In Eastern Europe, 2010 had an exceptionally warm summer, breaking the 500-year-long seasonal temperature records (Barriopedro *et al.* 2011), but 2011 was even drier. Understanding how cambial activity is affected by warm and dry periods is crucial to predict how forest will respond to the predicted climatic changes. In order to determine the influence of warm and dry summers on tree growth, the cambial phenology of maritime pine was studied in two years with a pronounced summer drought: 2010 and 2011. We have tested the hypotheses that 1) growth onset occurs earlier in warmer years, and 2) the end of xylogenesis is water dependent, stopping earlier in drier years.

4.2 Material and Methods

4.2.1 Study site and Climatic Data

The study site is described in detail in section *2.2.1*. For the present study, 33 dominant trees were selected, 25 for the diameter increment monitoring and 8 for the anatomical study. The same trees were monitored in the two study years. The climate is typically Mediterranean with a strong oceanic influence. Precipitation occurs mainly in autumn and winter and the summer is hot and dry (Figure 4.1). The monthly meteorological data (maximum and minimum temperature and total precipitation), was downloaded from the closest grid point to the study site in CRU (8.75 E; 40.25 N), for the period 1950-2011.

The Standardized Precipitation-Evapotranspiration Index (SPEI) was calculated to evaluate the joint effect of temperature and precipitation, by calculating the monthly difference between precipitation and potential evapotranspiration for the 1950-2011 period (Vicente-Serrano *et al.* 2010). SPEI was calculated using the *SPEI* package from the R freeware program (http://cran.r-project.org). SPEI is a standardized index with an average of zero and a standard deviation of one, and it can be compared with other SPEI values over time and space. The 2010 and 2011 SPEI’s were compared with the SPEI from 1950-2011, by calculating the difference between them, in order to identify periods of drought. A period of drought was characterized by a negative SPEI value (Vicente-Serrano *et al.* 2010).

4.2.2 Xylem formation

Wood samples were collected weekly from March to December 2010 [Day Of the Year (DOY) 62-349] and from March 2011 to February 2012 (DOY 62-409), using a Trephor. The samples were then processed following the procedure described in section *2.2.2*.

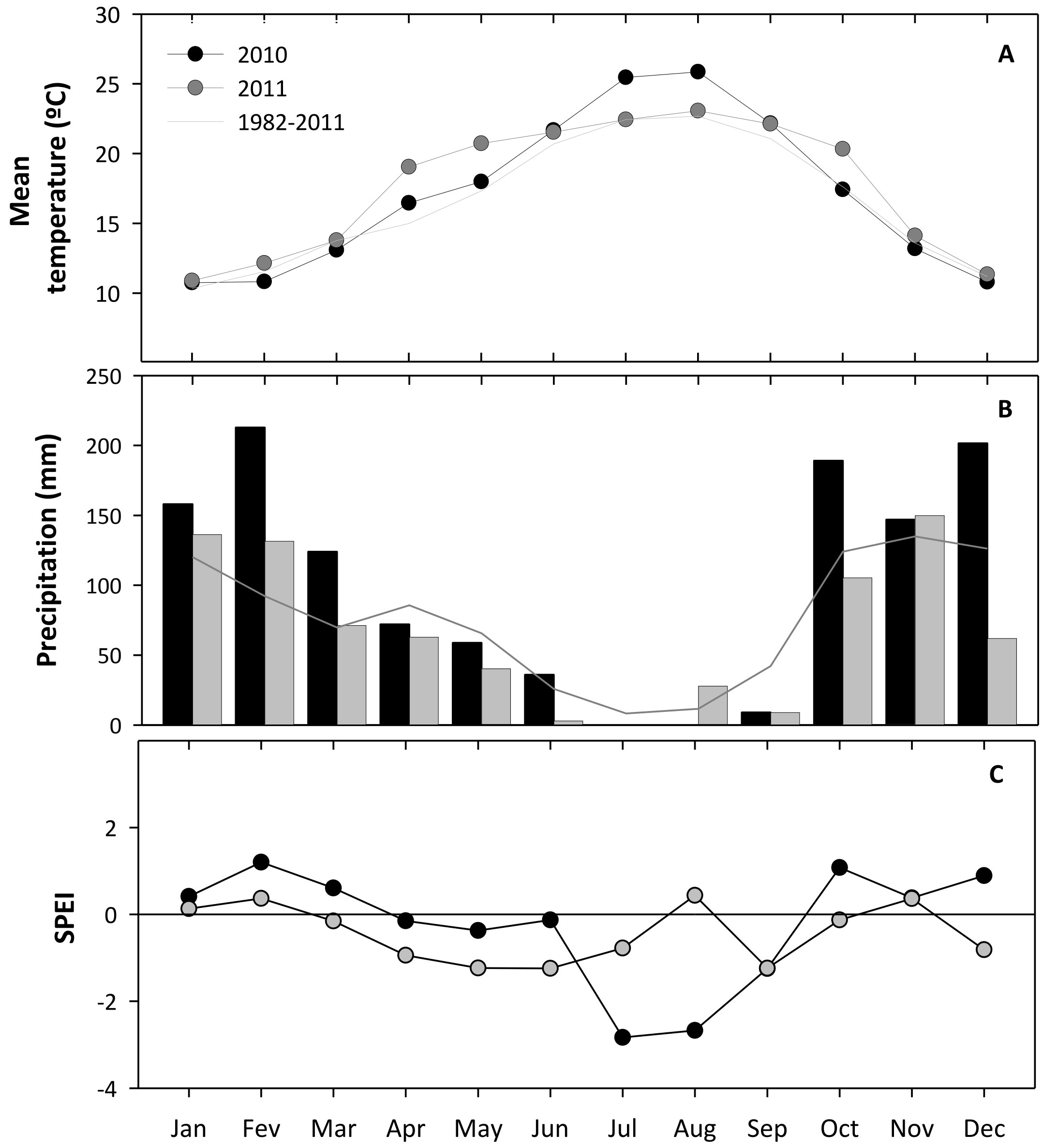

Figure 4.1 Temperature and precipitation from the nearest grid point (downloaded from CRU). (A) Mean monthly temperatures recorded in 2010 and 2011 and mean air temperature of historical series recorded from 1950 – 2011. (B) Sum of monthly precipitation recorded in 2010 (black bars) and 2011 (grey bars) and mean precipitation of historical series recorded from 1950 – 2011 (grey line). (C) Standardized Precipitation-Evapotranspiration Index [SPEI in 2010 (black circles) and 2011 (grey circles)]. The zero represents the index calculated for the long-term mean (1950-2011), the negative values are drought periods.

4.2.3 Xylem phenology

For each tree, the phenology of xylem development in 2010 and 2011 was compared. Two phenophases were considered: cell enlargement and cell wall thickening. The beginning of each phase was defined as the date when more than 50 % of the observed trees showed at least one cell in that phase. The end of each phase was defined as the date when less than 50 % of the observed trees presented at most one cell in that phase. The onset and end of enlargement and cell wall thickening were then ordered by date and a normal probability plot was used to compare the ordered date values with the percentiles of a normal distribution. All analyses were performed using SAS 9.2 (SAS Institute Inc., Cary, North Carolina).

4.2.4 Cell measurements

When xylem formation was complete in winter 2012, a microcore was collected per tree, embedded in paraffin and cut as described in section *2.2.2*. The resulting sections were stained with 1% aqueous safranin and permanently fixed with Canada balsam (Eukitt) to measure cell features using Wincell (Regent Instruments Inc.). A camera fixed on an optical microscope was used for image analysis, at 20 × magnification. On each section, three radial paths were selected to measure lumen area and cell wall thickness. Tracheids were classified in earlywood and latewood following the Mork's formula described in Denne (1989). The measures were averaged by year and the years were compared using a paired *t-test*.

4.2.5 Band dendrometers

To estimate the changes in stem diameter, band dendrometers made of astralon (model D1-L, UMS, Munich, Germany) were installed on 25 trees at breast height. This was done in January 2010 to allow for a period of adjustment before the beginning of the growing season (Linares *et al.* 2010). Before the installation of dendrometers, the bark was carefully removed with a chisel to better adjust the band dendrometer to the stem and to reduce non-xylematic sources of swelling and shrinking as much as possible (Zweifel *et al.* 2006). Band dendrometers were read weekly to the nearest 0.01 mm. To avoid biases due to the circadian rhythms of water storage and depletion, all measurements were done in early morning (Linares *et al.* 2009).

4.3 Results

4.3.1 2010 and 2011 climate

In 2010 and 2011, temperatures were higher than the long-term mean (1950-2011), except in January and December, when they were similar (Figure 4.1-A). The average annual temperature observed in 2010 and 2011 was 17.1 ^{o}C and 17.6 ^{o}C, respectively. The main differences between the two years were observed in April, May, July, August and October. April, May and October were warmer in 2011 and July and August in 2010. The long-term annual precipitation for the area was 965 mm. Total annual precipitation registered in 2010 and 2011 was 1200 and 800 mm, respectively. The higher precipitation recorded in 2010 was caused by extra precipitation in January, February, October and December (Figure 4.1-B). The precipitation in 2011 was similar to the long-term mean, except for the months of May, June, September and December, when it was lower. Both studied years showed periods of drought as confirmed by the negative SPEI values (Figure 4.1-C). In 2010 the drought period lasted from April to September, with prominent negative values in July and August, whereas in 2011, the drought period lasted from March to October. Although the SPEI for July was less negative (and even positive in August), spring and early summer were much drier in 2011 than in 2010.

4.3.2 Xylem phenology

Cambial phenology showed similar dynamics in 2010 and 2011 (Figure 4.2). In both years the cambium was already active on the first sampling date, in March, presenting 7 ± 2 and 9 ± 2 cells in the cambial zone in 2010 and 2011, respectively. The maximum number of cambial cells was observed during May-June in 2010, and March-April in 2011. At the end of summer (September) the number of cells in the cambial zone decreased to 5-7 cells in both years. Variation in the onset of cell enlargement was higher in 2011, with trees starting enlargement between DOY 68 and 117 (Figure 4.3). In 2010, 50 % of the studied trees had begun enlargement in DOY 85, whereas in 2011 the same observation was made earlier, in DOY 75. Enlargement also ended earlier in 2011, 50% of the trees had completed it in DOY 254 where in 2010 that was observed in DOY 288. The duration of enlargement, calculated as the difference between the onset and end, was 203 days in 2010, 24 days longer than in 2010. Cell wall thickening began earlier in 2011 with half of trees presenting tracheids in the cell wall thickening phase in DOY 90, compared with DOY 102 in 2010. The end of this phase was only observed in 2011, while in 2010 there were still tracheids in cell wall thickening at the end of the sampling season (DOY 345). According to the normal distribution, in 2011 the end of cell wall thickening for 50 % of the trees was on DOY 355, which means that, on average, it lasted 265 days. Regarding cell production, more tracheids were produced in 2010 than in 2011 (Figure 4.4). In the end of the 2010 there were 40 mature xylem cells and in the end of 2011 there were 25. Additionally, in 2010 the total number of cells showed a faster growth rate (Figure 4.4).

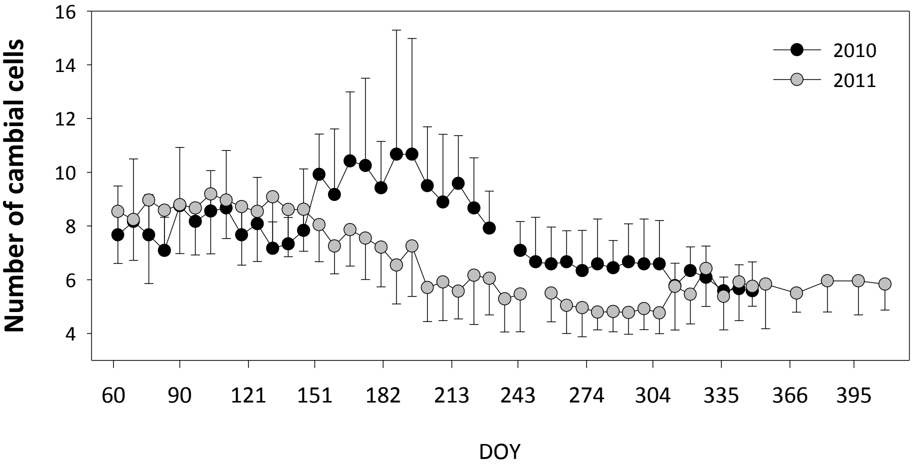

Figure 4.2 Number of cells in the cambial zone of *Pinus pinaster* during 2010 and 2011. Vertical bars represent standard deviation.

4.3.3 Xylem characteristics

The number of earlywood tracheids and their characteristics were similar in both years with no significant differences in the studied variables (number of tracheids, $t = 0.89$, $P = 0.42$; lumen area, $t = -0.37$, $P = 0.72$ and cell wall thickness, $t = 2.24$, $P = 0.06$) (Figure 4.5). Latewood presented a higher number of tracheids in 2010 ($t = 3.58$, $P = 0.01$) with a larger lumen area ($t = 2.41$, $P = 0.05$) but a similar cell wall thickness ($t = 0.05$, $P = 0.96$). The number of earlywood cells varied between 12 and 14, whereas latewood presented an average of 16 and 13 cells in 2010 and 2011, respectively. The lumen area was larger in earlywood (1200 – 1250 µm^2) than in latewood (140 – 180 µm^2) and the opposite was observed in cell wall thickness (3.5 – 4 µm and 7 µm in earlywood and latewood, respectively).

4.3.4 Stem radius variations

In both years, stem diameter increments showed a clear bimodal pattern, characterized by a pronounced first period of increment in spring, followed by plateau in summer, and a second less marked period of increment in autumn (Figure 4.6). Stem diameter increments showed a high synchrony in the two years, with the same onset date around DOY 75. The maximum increment was reached at the end of April (DOY 112) in 2010 and 10 days earlier in 2011 (Figure 4.6-B). After the maximum, the increment started to decrease, reaching a variation close to zero in August-September (DOY 180 – 250). The second increment peak was observed in October-December (DOY 250 – 340), starting earlier in 2010 (DOY 286) than in 2011 (DOY 306). The total cumulative increment observed in 2010 was higher than in 2011 (Figure 4.6-A).

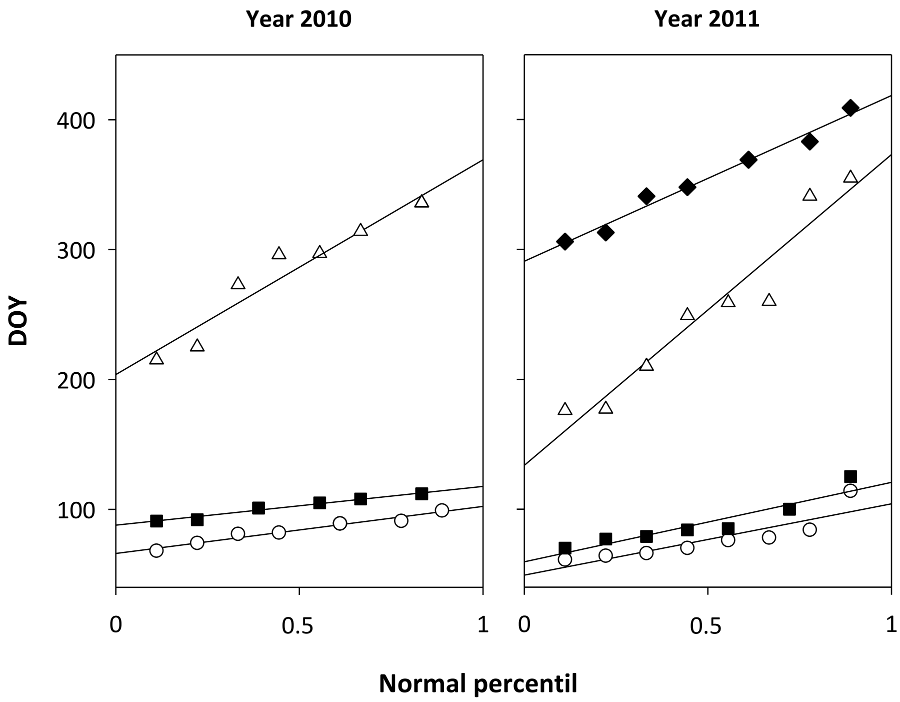

Figure 4.3 Distribution of the onset of cell enlargement (open circle), end of enlargement (open triangle), start of cell wall thickening (closed square) and cessation of cell wall thickening (closed diamond) for 8 trees of *Pinus pinaster* in 2010 and 2011 expressed in days of year (DOY).

4.4 Discussion

This study investigated timings and duration of cambium phenology and dynamics of xylem growth in maritime pine in two warm and dry years (2010 and 2011). Our findings confirmed the double climatic control of xylogenesis in the Mediterranean region: cambial activity started earlier in response to a warmer late winter and stopped earlier in the drier year, confirming the hypotheses. Although cambial activity started earlier in 2011, it also stopped earlier, demonstrating that onset and duration of cambial activity are independent. The drier spring and summer triggered an earlier start of latewood formation, resulting in a smaller tree-ring and narrower tracheids.

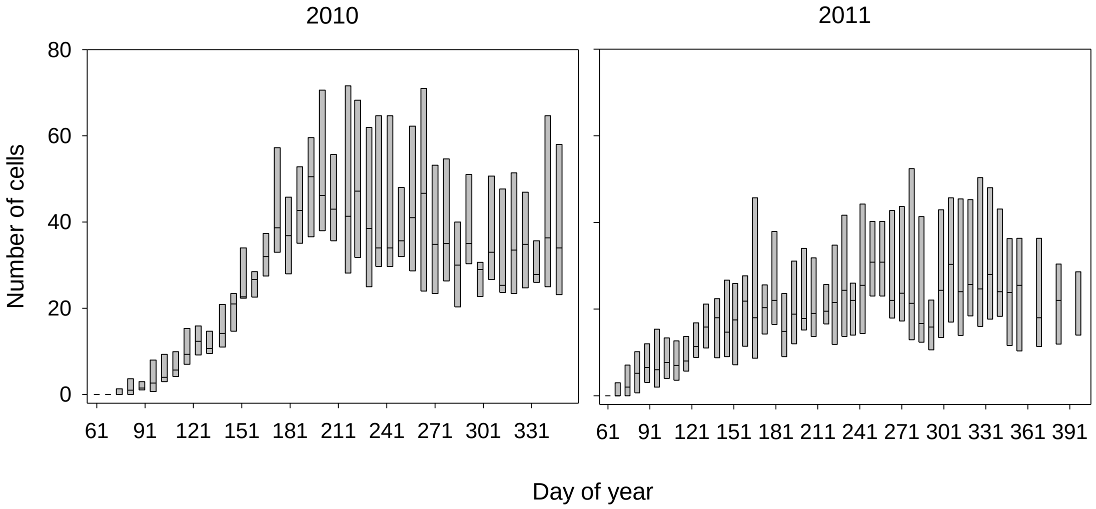

Figure 4.4 Variation in the total number of cells (cells in enlargement, wall thickening and mature) during 2010 and 2011. The horizontal middle line represents the median.

4.4.1 Cambial onset

The minimum temperature required for tree growth in species of very cold environments is between 0 and 10 ºC (Korner 1998; Deslauriers and Morin 2005; Rossi *et al.* 2008). The range of winter temperatures experienced in the study site was within those threshold values, however, the cambium was dormant. Nevertheless, an earlier onset of cambial activity was observed in the warmer year (2011), suggesting that the cambium was responsive to temperature. The fact that the cambium was dormant although the temperature was above the threshold, suggests that probably, the temperature threshold in the Mediterranean (and other warm regions,) is higher than the one established for altitude and Boreal environments. Factors other than or in addition to temperature can also trigger the break of winter dormancy in trees, such as photoperiod (Nizinski and Saugier 1988; Rossi *et al.* 2006). In winter, the short day length slows down the metabolism in the meristems, triggering the tree to enter a dormant state until the return of favorable environmental conditions (Rossi *et al.* In press). Our observations confirmed that although winter temperature was above the described temperature threshold, cambial onset was controlled by temperature, occurring earlier in warmer years. Several studies have demonstrated that cambial activity is highly responsive to temperature, starting earlier in years with higher spring temperature (Begum *et al.* 2008; Deslauriers *et al.* 2008; Rossi *et al.* 2008).

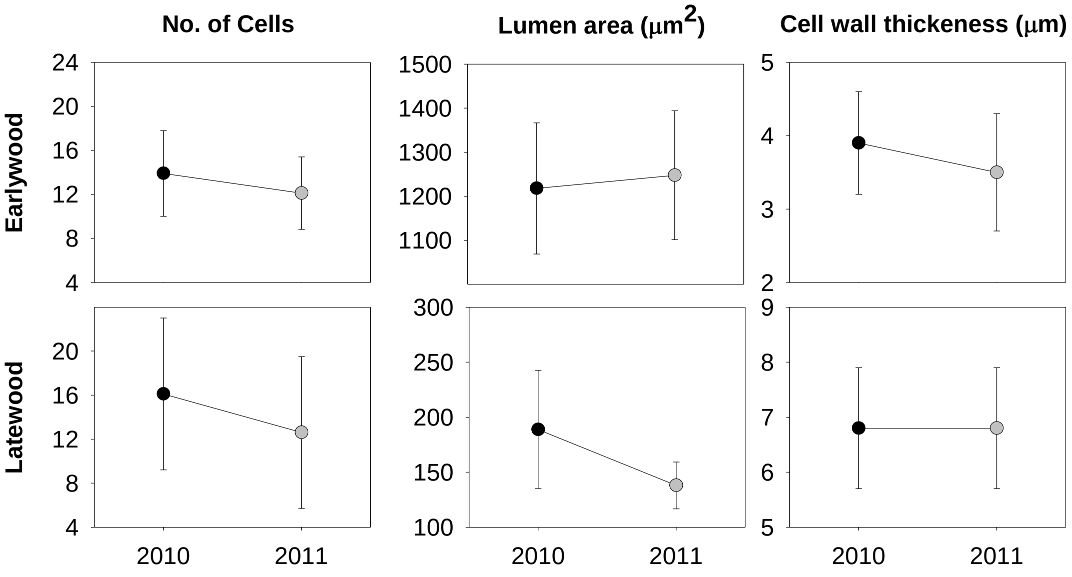

Figure 4.5 Early- and latewood number of cells, cell area (µm) and cell wall thickness (µm^2) in 2010 (black circles) and 2011 (grey circles). Vertical bars represent the standard deviation.

4.4.2 Impact of drought on xylem development

Although cell differentiation started later, the tree ring formed in 2010 was wider than that of 2011. Thus, an earlier onset of cambial activity did not result in a larger tree ring or a longer duration of xylogenesis as reported by Lupi et al. (2010) in the boreal forest of Quebec. Dendrochronological studies on maritime pine have shown that radial growth is closely related to April and May precipitation (Vieira *et al.* 2009; Campelo *et al.* 2013). During 2011, April and May precipitation were below average, especially in May. In addition to lower spring precipitation, temperatures in April and May were 4.4 ^{o}C above the long-term mean. Thus the spring period in 2011 was much drier when compared to 2010, as clearly shown by the more negative SPEI values. The warmer late winter and drier spring observed in 2011 displaced maximum cell production in time, from June to April, demonstrating that wood formation adjusts to the prevailing climate conditions. Timings of xylogenesis were triggered by climate, with no relation being observed between onset and end of xylogenesis. Water stress is the primary factor limiting tree growth in the Mediterranean area, thus an earlier onset of the growing season would only trigger a wider ring if no water stress occurred during spring.

Water stress causes severe limitations to plant growth and metabolism, which translates into growth reduction and even tree mortality (Allen *et al.* 2010; Choat *et al.* 2012). During periods of drought, turgor-driven cell expansion, cell wall synthesis and protein synthesis are substantially affected (Hsiao *et al.* 1976; Chaves *et al.* 2002). Trees respond to water stress by adopting one of two behaviors: isohydric or anisohydric (McDowell *et al.* 2008). Maritime pine adopts an isohydric behavior, which means that it will close its stomata in order to avoid hydraulic failure (Ripullone *et al.* 2007). By closing the stomata, carbon uptake and photosynthesis are affected, which translates into a decrease of carbohydrate availability, generally followed by a growth reduction (Oribe *et al.* 2003; Michelot *et al.* 2012) and ultimately carbon starvation induced mortality (McDowell 2011; Adams *et al.* 2013). In an experimental study, using an isohydric species (*Pinus edulis* Engelm.), Adams et al. (2009) showed that warmer conditions (≈4 ºC) triggered a faster consumption of carbon reserves, increasing the risk of mortality due to carbon starvation. The summer of 2010 was exceptionally warm in Europe (Barriopedro *et al.* 2011), while the drought period in 2011 was longer. The cumulative effect of two consecutive hot and dry years probably had a negative impact on the carbon reserves of maritime pine, which reflected in the narrower tree ring formed in 2011. The transition from early- to latewood reflects the availability of water for cambial activity (Domec and Gartner 2002). Lower water content decreases cell turgor thus affecting cell enlargement resulting in smaller, denser and mechanically stronger tracheids (Abe *et al.* 2003; Sperry *et al.* 2006). Although there was a higher number of latewood tracheids in 2010, latewood formation started earlier in 2011. Besides the differences in the number of latewood tracheids, their anatomical characteristics also differed, with the 2011 tracheids having a smaller lumen area. The formation of tracheids with smaller lumen area and thicker cell walls diminishes the hydraulic conductivity but increases the xylem resistance to drought-induced cavitation (Hacke and Sperry 2001; Dalla-Salda *et al.* 2009). Numerous studies have linked higher wood density in conifers with drought stress (Domec and Gartner 2002; Pittermann *et al.* 2006). In a water exclusion experiment in the field, Belien *et al*. (2012) observed that the number of tracheids was unaffected by the treatment, but the lumen area of tracheids was reduced in trees subjected to rain exclusion. In the present study, a decrease was also observed in the lumen area of latewood tracheids in response to water stress.

4.4.3 Diameter increment variations

Our study revealed a close relationship between the rapid increment in stem diameter and the beginning of the enlargement phase in both years. However, cambial onset could not be accurately determined by dendrometers (Linares *et al.* 2009; Camarero *et al.* 2010). The resolution of band dendrometers is probably not sufficient to record the microscopic variations caused by the first increases in cambial cell number (Gruber *et al.* 2009). Increment in diameter consists of several components including the daily swelling and shrinking of the outermost stem tissues due to water absorption and thermal variations, which makes it difficult to isolate the wood formation component (Zweifel *et al.* 2006). In maritime pine there were two maximal increments in both years: one in spring and another in autumn. This bimodal pattern of stem radial increment is characteristic of trees growing under a Mediterranean climate and follows water availability (Cherubini *et al.* 2003; Linares *et al.* 2009; Camarero *et al.* 2010).

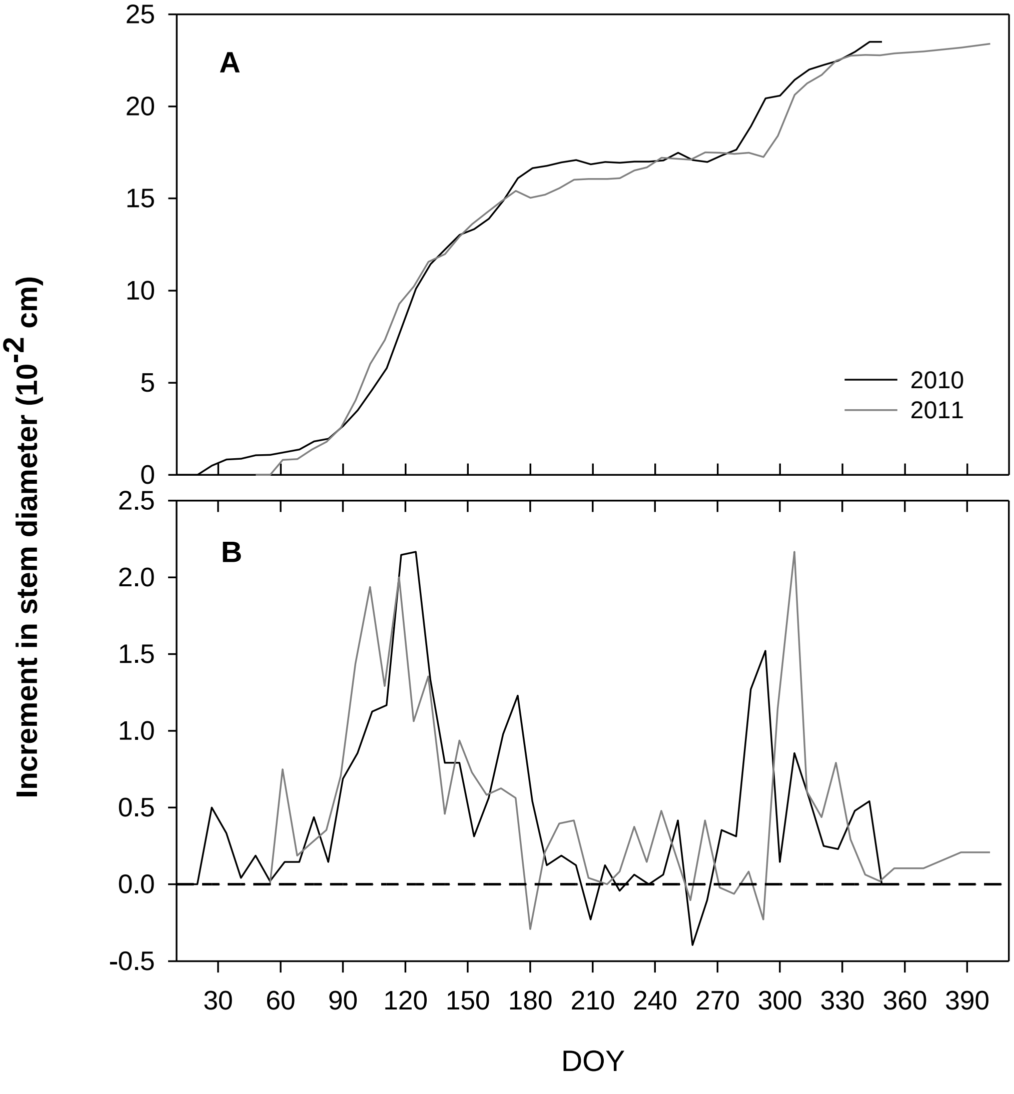

Figure 4.6 Cumulative (A) and weekly increments (B) in the stem diameter measured with band dendrometers in 2010 (black line) and 2011 (grey line).

In spring, the increase in stem diameter presented a delay between years (earlier in 2011), corresponding to the observed differences in the beginning of cell enlargement. The spring peak was observed in April, in both years, two months before the summer solstice. Several authors have reported maximum growth rates around the summer solstice in conifers of cold environments (Rossi *et al.* 2006; Gruber *et al.* 2010). Rossi (2006) hypothesized that the maximum growth rates of trees growing at altitude synchronize with maximum day length and not temperature to ensure that tracheid differentiation is safely completed before winter. In the study area, however, the average winter temperatures were never below the minimum temperature range for tree growth, allowing tracheid differentiation to continue all year round. Actually, in 2010 cell wall deposition was not complete in mid-December, when the sampling ended (*Chapter II*). De Luis (2007) made similar observations on Aleppo pines growing in Spain, suggesting that cell wall deposition is not limited by winter temperatures in the Mediterranean region.

During the summer, when temperatures are higher and there is a decrease in water availability, the stem dehydrates and contracts (Zweifel *et al.* 2001; *Chapter V*). In the Mediterranean climate, rainfall events after the summer can trigger the formation of new cells by the cambium and/or the differentiation of immature cells, which can result in the formation of intra-annual density fluctuations (Campelo *et al.* 2007). Although enlargement and differentiation of immature cells was observed in the autumn of 2010, probably produced by the cambium during early summer (*Chapter II*), the second increment peak corresponded mostly to a rehydration of the stem. The 20 days delay between years in the autumn increment period (later in 2011) corresponded to the differences in the precipitation events that triggered stem rehydration.

4.5 Conclusion

Maritime pine was responsive to late-winter conditions, starting cambial activity and xylem differentiation earlier in response to warmer temperatures. Although growth differentiation started earlier in 2011, it also ended earlier due to drier conditions during spring, thus resulting in a narrower tree ring. Our results clearly demonstrated the influence of precipitation on xylogenesis, with lower water availability in April and May restricting cell production. In addition, a longer drought period had repercussions not only on the number of latewood tracheids (fewer) but also on their anatomy, with cells presenting a smaller lumen area. Maritime pine xylogenesis is thus under a double climatic control, temperature controls growth onset and water availability determines growth cessation. Band dendrometers proved to be useful to estimate the onset of the cell enlargement phase. Although a bimodal increment pattern was recorded by the dendrometers, the autumnal increment period was mostly rehydration of the stem.

CHAPTER V

Seasonal and daily cycles of stem radial variation of *Pinus pinaster* in a drought-prone environment

5.1 Introduction

The variation in stem size of trees results from an irreversible component due to growth and a reversible component due to changes in the water balance of tissues (Irvine and Grace, 1997). As a general rule, the stem contracts during the day, due to transpiration and photosynthesis and expands during the night and on rainy days when water reserves are gradually replenished. Cermak *et al.* (2007) observed that the internal water storage can provide a significant proportion of the total diurnal and even seasonal water used by a plant. The water stored in the elastic tissues of the stem buffers the lag between roots and shoot, thus preventing embolisms and ensuring optimal transpiration rates (Peramaki *et al.* 2005). The dynamics of the daily changes in stem size can thus provide valuable information about the water status and radial growth of trees (Downes *et al.* 1999; Zweifel *et al.* 2000; Deslauriers *et al.* 2003; Deslauriers *et al.* 2007; Turcotte *et al.* 2009). In fact, stem radial variation can be de-trended in order to extract the tree water deficit, (Zweifel *et al.* 2005; Drew *et al.* 2011).

It is well established that timing and magnitude of daily variations in stem size are mainly determined by the course of transpiration and soil water content (Kozlowski 1976; Zweifel *et al.* 2006). So, it is expected that these daily cycles are strictly dependent on microclimatic conditions and can quickly change according to weather conditions. Dendrometer data collected in the Swiss Alps by King *et al.* (2012) demonstrated that the amplitude and duration of the circadian cycles of Norway spruce (*Picea abies* Karst.) and European larch (*Larix decidua* Mill.) changed during the year. Smaller cycles were observed on rainy days and larger ones when the daily mean temperature was between 15 and 20 °C. Duchesne and Houle (2011) studied the daily variation in stem radius of balsam fir [*Abies balsamea* (L) Mill.] growing in the boreal forest of Quebec and observed that stem expansion was higher on rainy days. In the Italian Alps, an eight year study on the stem radial variation of timberline conifers found that stem increment responded positively to precipitation and negatively to temperature (Deslauriers *et al.* 2007). All the above-mentioned studies were performed in cold environments with a short growing season, where water availability is not generally a problem. Are the daily and seasonal changes of stem diameter the same in a drought prone environment, with a longer growing season, like the Mediterranean climate? If not, how do trees respond to water stress?

Seasonal variations in stem radius have already been studied in the Mediterranean region with contradictory findings. Gutierrez *et al.* (2011) reported that *Quercus ilex* L. radial increment was mainly constrained by temperature, whereas Camarero *et al.* (2010) observed that in

Juniperus thurifera L. and *Pinus halepensis* (Miller) the climatic response changed during the year, with stem radius variations dependent on temperature during growth onset and on precipitation during the summer. A different study revealed that *Abies pinsapo* (Boiss.) wood formation was greatly reduced by drought (Linares et al., 2009). Although radial increment has already been studied on a seasonal level, the daily variations of stem radius still remain undetermined under a Mediterranean climate. In this study the seasonal and daily variations in the stem radius of maritime pine (*Pinus pinaster* Ait.), growing on a drought prone site in Portugal are described. The following hypotheses were tested 1) changes in stem size have a seasonal and daily pattern that reflects the availability of water and 2) once the internal water storage is depleted the tree enters a quiescent state.

5.2 Methods

5.2.1. Study site

The study site is described in *section 2.2.1*.

5.2.2. Data collection

From January to November 2010, automatic band dendrometers (EcoMatik, model DC, Munich, Germany) were used to measure stem perimeter variations in four trees with similar characteristics in terms of dominance, vigor and tree-ring width (sampled at the same time as the trees in *Chapter II*). The dendrometers were installed on the stem at a height of about 3 m, 1 month before the start of the growing season, to allow a period of adjustment. Before installing the dendrometers, the outermost tissues of the bark were removed to reduce the influence of hygroscopic swelling and shrinkage of the bark, and to ensure close contact with the xylem. Plastic beads were placed around the dendrometer cable to reduce friction with the tree bark. The dendrometer sensors were linear variable differential transducers with an accuracy of 7 µm enclosed in an aluminum frame and attached to the tree via a stainless-steel cable. Sensors and cables have a thermal expansion coefficient inferior to 0.1 and 1.4×10^{-6} µm C^{-1}, respectively. Measurements were collected every 20 min and stored in a datalogger (The ulogger 4R, EcoMatik, Munich, Germany). Data were transformed into radial measurements by dividing the circumference by 2π, and hourly averages were calculated for the following analyses. The final dendrometer series presented missing data due to technical problems that occurred during the monitoring period, and outlier values, that were removed.

Daily maximum and minimum temperatures and total precipitations were acquired from the nearest meteorological station belonging to the Instituto Português de Meteorologia and located in Figueira da Foz, at 25 km South from the study site.

5.2.3. Extraction of stem variation phases

The time series obtained from each tree were individually processed according to the stem cycle approach of Downes *et al.* (1999), modified by Deslauriers *et al.* (2003). Cycle extraction was performed using a three-step procedure composed of two SAS routines (SAS Institute, Inc.) specially developed to analyze hourly automatic dendrometer data (Deslauriers et al., 2011). The procedure divides the series into three distinct phases: (1) contraction, the period between the first maximum radius and the next minimum; (2) recovery, the period from the minimum until the position of the previous maximum value or when the stem reverts to a contraction phase; and (3) radial increment, which can be positive or negative depending on whether or not the previous maximum was achieved (Figure 5.1). A cycle was constituted by a contraction, followed by a recovery and a radial increment phase, when present. For each cycle, the SAS routines calculated the amount of stem radial variation and its relative duration.

In order to better describe stem radial variation over the year, the dendrometer series were divided into five periods according to the amplitude of the cycles and net radius variation (Turcotte et al., 2009): period 1, winter dormancy during which the radius variation was around zero [Day Of the Year (DOY) 20-77]; period 2, spring growth, from the start of positive radius increment (when the daily increment was higher than in the previous day), until the spring maximum (DOY 78-178); period 3, pre-summer contraction, when the daily increment was negative (DOY 179-214); period 4, summer dormancy, when the amplitude of the cycles reached minimum values (DOY 215-275); and period 5, autumn re-hydration, during which the radial increment increased rapidly in a short period of time (DOY 276-328; Figure 5.2). Stem radial variation was also averaged per hour in order to compare its daily amplitude in the five periods.

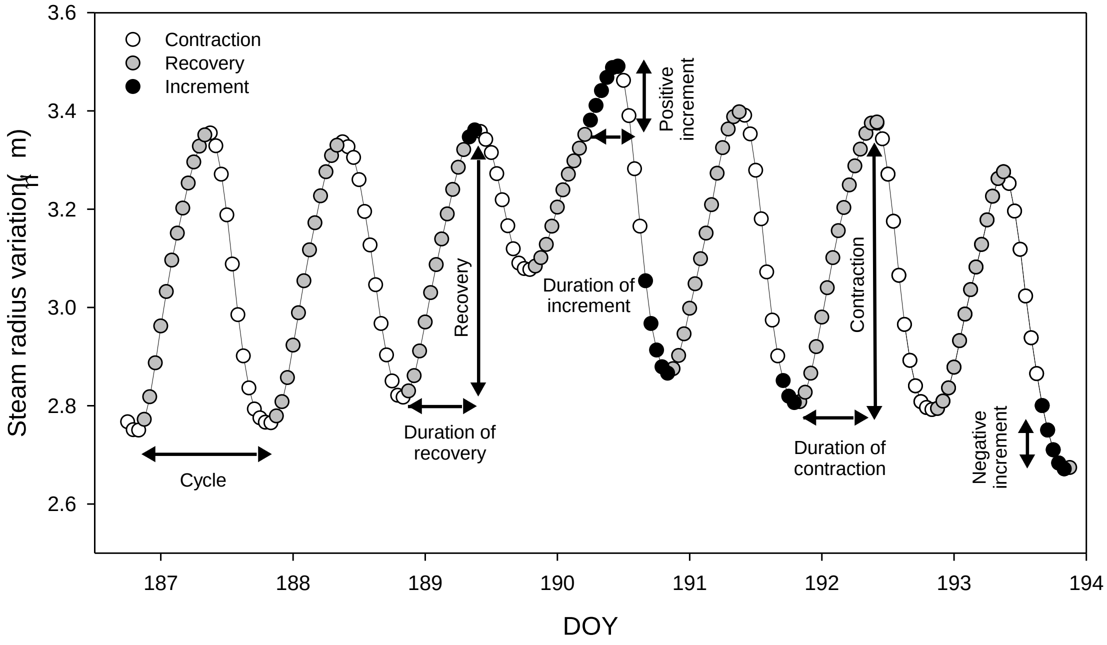

Figure 5.1 Cycles of stem radius variation detected by automatic dendrometers in maritime pine. The cycles are divided in three phases: 1) recovery, period between the first minimum and the previous maximum; 2) contraction, period between the maximum and the next minimum; and 3) increment, period when the stem radius exceeds the previous maximum until the subsequent maximum. Circles represent hourly data measured in July 2010.

5.2.4. Tree water deficit

To distinguish changes in stem radius due to water content variability and growth, a de-trending approach was used as described in Zweifel *et al.* (2005). Tree water deficit (Δ*W*) was calculated as the difference in stem size under increasingly dry conditions relative to the size of the fully-hydrated stem (Figure 5.3). We assumed that at the beginning of the study the stem was fully hydrated, thus presenting a zero value of Δ*W.* Increasingly negative values indicate increasing tree water deficit.

5.2.5. Statistical analyses

The three phases of stem radial variation were re-scaled at an average of zero (by subtracting the mean from each value) and linear regressions were calculated between amplitude and duration of each phase. Data of temperature and precipitation were processed following the occurrence of each cycle. Maximum and minimum temperatures and total precipitation were calculated for each circadian cycle and compared with the corresponding radial variations. Pearson correlations were performed separately for the five periods to determine the influence of weather on the amount of stem variation and duration of each phase.

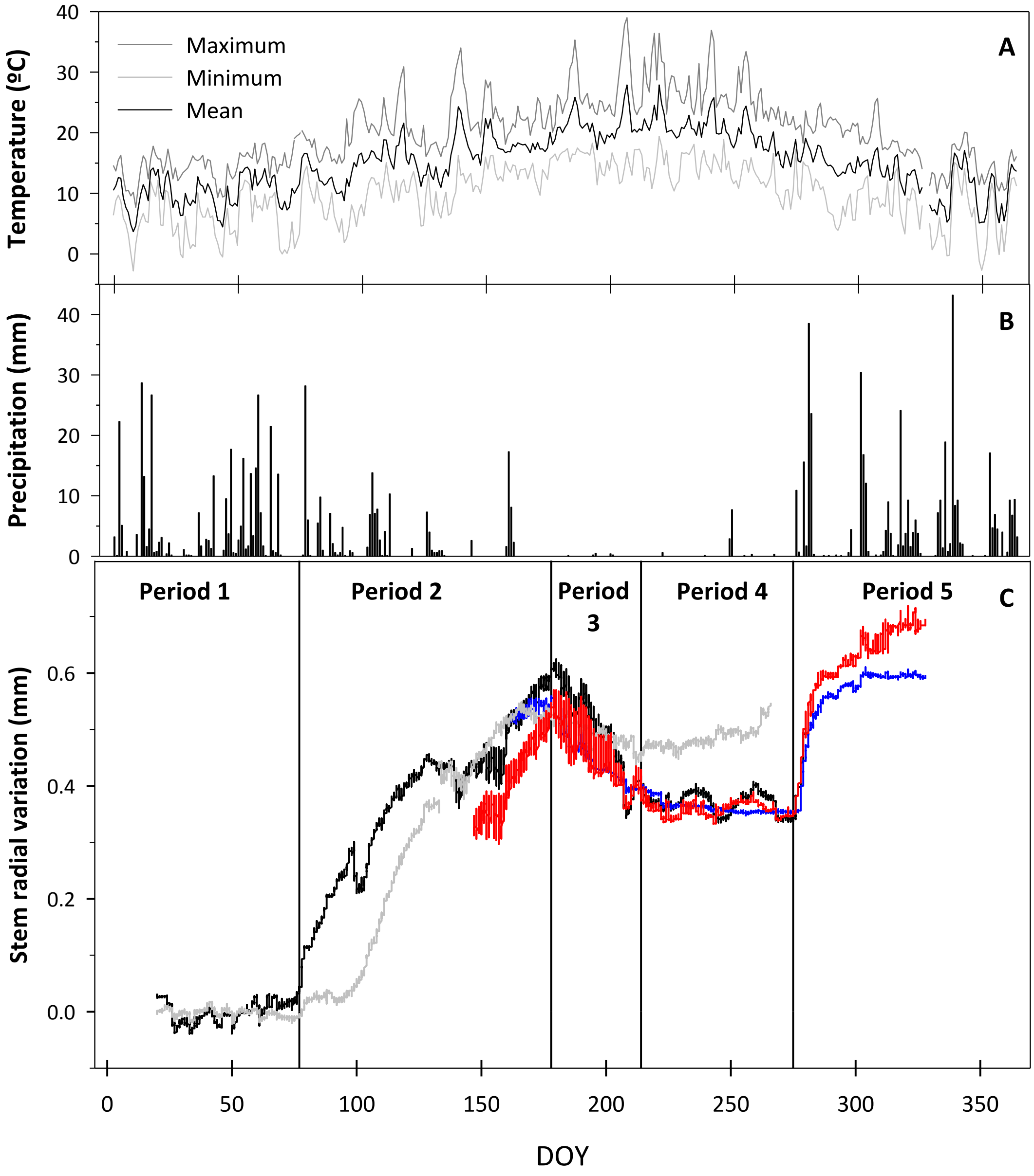

Figure 5.2 Temperature and precipitation (A and B, respectively) in Figueira da Foz (data from Instituto Portugues de Meteorologia) and stem radial increment of maritime pine in 2010 (C); the vertical lines delimit the 5 periods of increment during that year: Period 1: winter; Period 2: spring; Period 3: pre-summer; Period 4: summer; Period 5: autumn.Figure 4. Mean hourly variation in stem radius in the five periods of 2010. A) raw data; B) cumulative data.

Missing data and the different lengths of the selected periods could affect the robustness of statistical tests. To address this issue, tests were validated using the bootstrap procedure (Efron and Tibshirani 1993). Regressions and correlations were repeatedly calculated by randomly re-sampling the original data set and estimating the confidence intervals of the distribution. Bootstrapping was performed 10,000 times to improve the robustness of the results (e.g. correlation coefficient or regression slope), which were considered significant when both confidence intervals were either higher or lower than zero.

5.3 Results

5.3.1. Temperature and precipitation in 2010

Mean daily temperature in the winter prior to the growing season ranged between 3 and 13 °C. The minimum temperature only dropped below 0 °C on three occasions, on January 9th and 29th, and February 14th (Figure 5.2-A). In spring and summer, temperatures rose reaching a maximum of 39 °C in mid-July. In September, they gradually started to decrease, but mean temperatures were still above 15 °C. Temperatures only dropped below 10 °C in mid-November. Precipitation was more frequent in January-March and October-November (Figure 5.2-B). In April precipitation started to decrease and very few precipitation events were observed from June to September, with a total of 40mm registered during those months. The first precipitation in autumn occurred at the beginning of October, with a total of 90 mm in one week.

5.3.2. Stem radial variation and tree water deficit

Stem radius variation presented a clear seasonal pattern with marked characteristics in each period (Figure 5.2-C). Period 1 showed daily cycles of stem radius but no positive increment. Continuos positive radial increments were first observed in period 2. Vigorous growth was observed during this period, which lasted until the end of June, when a maximum was reached. In period 3, stem radius decreased markedly until the beginning of August. In August and September (period 4) the stem radial variation was minimal. In October (period 5), stem radius increased drastically within 10 days, which coincided with the first significant precipitation after the summer drought (Figure 5.2-B). Stem radial variation started to stabilize in November.

The variation observed in the tree water deficit was the opposite of that observed in stem radius (Figure5.3). In periods 1 and 2 the trees recovered during the night from the ΔW generated during the day (returning to zero). During period 3 there was a general decline of ΔW with trees not able to recover to a ΔW close to zero on a daily and monthly basis, resulting in the shrinkage of the stem. The ΔW remained negative during period 4 (summer). The stem only recovered the initial hydration status in period 5.

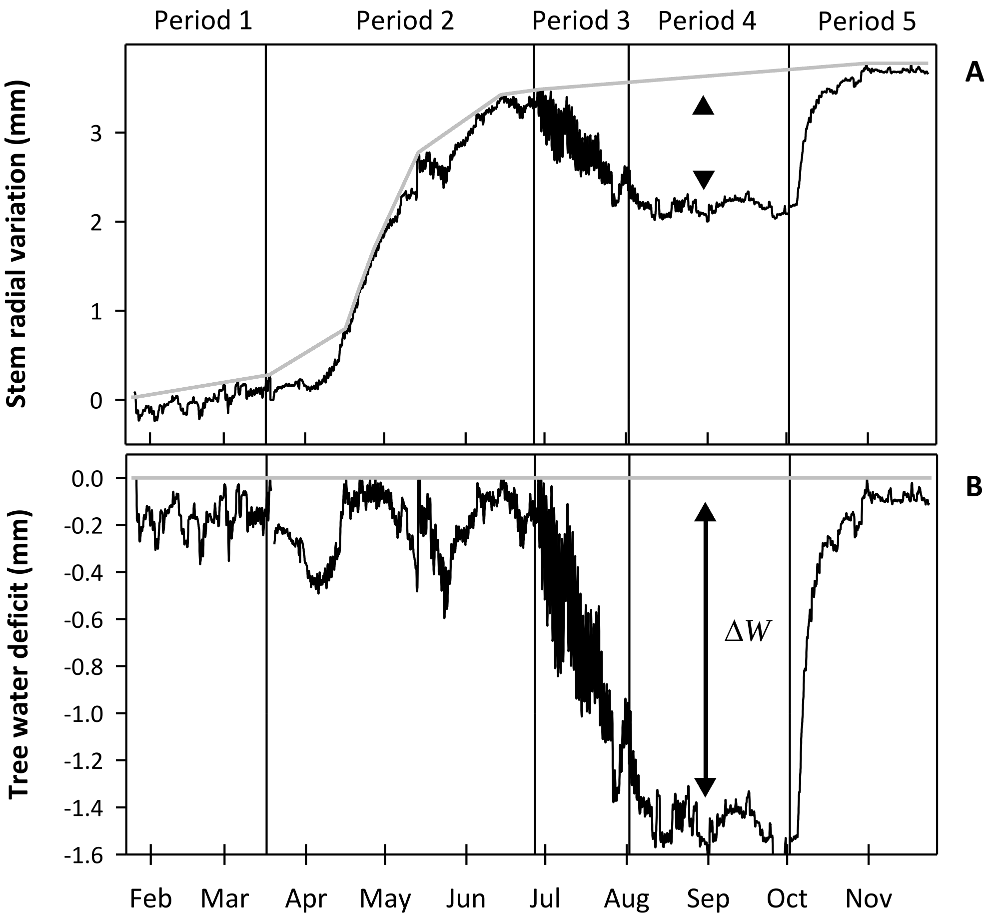

Figure 5.3 De-trending of the stem radial variation of Maritime pine (*Pinus pinaster*) from February to November 2010. A) Stem radius variation (black line) and growth trend line (red line); B) Tree water deficit (ΔW); the vertical lines delimit the 5 periods of increment during that year: Period 1: winter; Period 2: spring; Period 3: pre-summer; Period 4: summer; Period 5: autumn.

Besides the seasonal pattern, stem radius variation changed considerably during a 24-h period (Figures 5.1 and 5.4). The average maximum radius was observed between 09:00 and 13:00 and the minimum between 19:00 and 21:00 (Figure 5.4-A). The largest daily stem radius variation was observed in period 3, with variations of up to 0.4 mm. Although there was a circadian rhythm of contraction and recovery during period 1, the cycles started and ended at the same radial value, suggesting that no increment occurred (Figure 5.4-B). In fact, continuous positive radial increments were only observed in period 2 and 5 (Figure 5.4-B). On the contrary, in periods 3 and 4, the mean values at the end of the day were lower. The percentage of days with positive increment was also different in each period with periods 2, 5 and 1 presenting the higher percentage (71, 60 and 52%, respectively) and periods 3 and 4 the lowest (33 and 44%, respectively).

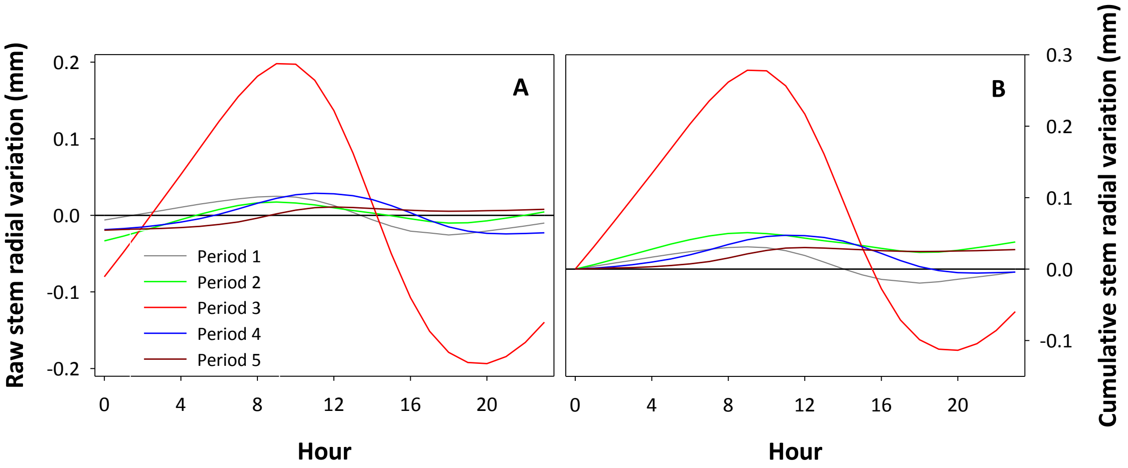

Figure 5.4 Mean hourly variation in stem radius in the five periods of 2010. A) Raw and B) cumulative stem radial variation (mm).

5.3.3. Seasonal changes in cycle duration and amplitude

The cycles presented different amplitudes and duration of their phases within every period (Figure 5.5). Contraction and recovery phases varied between 0 and 0.6 mm and the increment ranged between -0.13 and 0.1 mm. Period 3 showed the highest amplitude of contraction and recovery whereas the opposite was observed in period 5. Except for periods 1 and 5, recovery lasted longer than contraction. Increment was generally positive in periods 2 and 5, negative in period 3, while varying around zero in the remaining periods. The duration of increment decreased from period 1 and reached a minimum in period 3, increasing again in periods 4 and 5.

Regression analyses revealed some significant linear relationships between amplitude and duration of the phases (Figure 5.6). In period 3, the amplitude of recovery and increment was highly dependent on duration, and the same was observed in periods 4 and 5 for contraction. The distribution of the duration of phases was similar in periods 1 and 5 (Figure 5.6, pie charts). In periods 2 and 3, contraction had a longer duration than recovery and increment represented only 26% and 12% of the cycle, respectively. In period 4 the recovery lasted longer than the contraction phase.

5.3.4. Responses to weather

The amplitude and duration of stem radial variation presented different responses to temperature and precipitation throughout the year (Figure 5.7). During period 1 (winter dormancy), a positive correlation was observed between maximum temperature and the amplitude of contraction and amplitude and duration of recovery. Period 2 (spring growth) showed similar results although a negative correlation with precipitation was also observed.

Period 3 (pre-summer contraction) exhibited a positive response of amplitude of contraction and recovery to minimum temperature and a negative response of amplitude of contraction to maximum temperature. A negative response to minimum temperature was also observed in the duration of contraction. During the pre-summer contraction duration of increment also responded negatively to precipitation. In period 4 (summer) amplitude of contraction and recovery was positively correlated with minimum and maximum temperatures, respectively. A positive response was also observed between duration of contraction and recovery and maximum temperature. In period 5 (autumn), increment showed a positive correlation with minimum temperature.

5.4. Discussion

5.4.1. Seasonal variation of stem radius and tree water deficit over the year

The study investigated stem radius variations of maritime pine growing under a Mediterranean climate by identifying five periods of distinct physiological activity: winter dormancy, spring growth, pre-summer contraction, summer quiescence and autumn re-hydration. Radial increment started in spring and reached its maximum in June. A marked contraction was observed in summer, followed by a period of stable fluctuations. In autumn, after the first rains, the stem re-hydrated rapidly. At daily resolution, the cycles of radial variation changed in amplitude and duration during the year, with the largest variations exhibited in summer, when the amplitudes were 10 times higher than those observed in the other periods.

In trees of cold environments, stems show a marked re-hydration before the beginning of growth (Tardif *et al.* 2001; Deslauriers *et al.* 2003; Turcotte *et al.* 2009), but this was not observed in maritime pine at the study site. Re-hydration is a direct consequence of the freezing temperatures: at high altitudes and latitudes, stem size closely follows the daily changes in winter temperature rather than the tree evapotranspiration, with shrinking and swelling occurring during the night and day, respectively, producing what is called an inverted cycle (Zweifel and Hasler 2000). Ultimately, water is withdrawn from the living cells to avoid freeze-induced cavitation and, consequently, the stem diameter reduces (Zweifel and Hasler 2000). Prior to spring growth, when temperatures rise above freezing point, the ice melts and water can replenish the living tissues (Turcotte *et al.* 2009). This swelling of the tissues restores the tree to a physiologically active state. In our study site however, there were no inverted cycles in winter indicating that the sap did not reach freezing point. In fact, temperatures only dropped below 0 °C occasionally and for short periods.

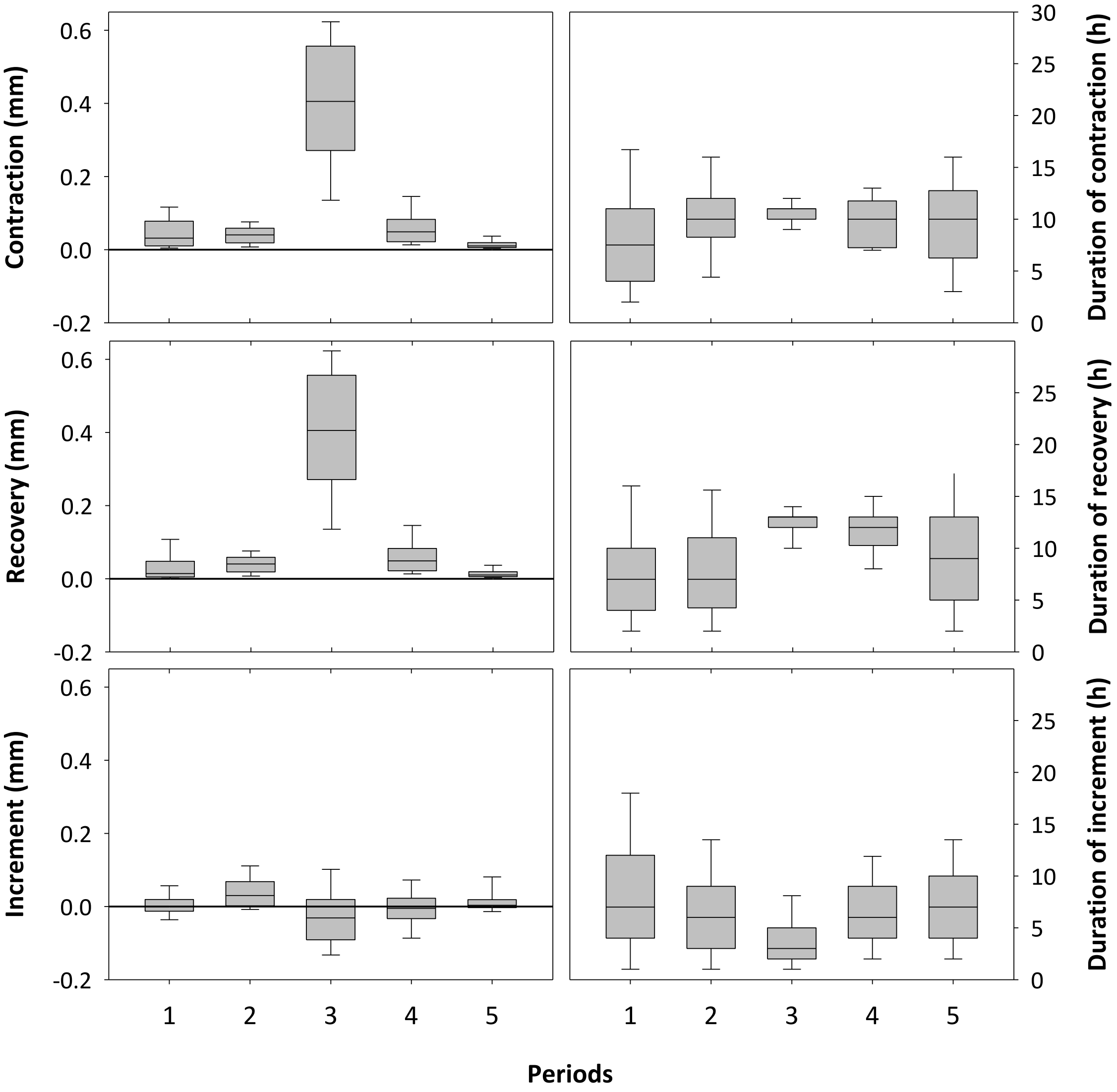

Figure 5.5 Amplitude and duration of the three phases of stem radius variation.

Marked stem contractions and re-hydrations were observed in summer and autumn, respectively. In July, when the highest temperatures and day length were registered in the site, the circadian cycles of stem contraction and recovery showed amplitudes 10 times higher than in the rest of the year. A similar finding was made by King *et al.* (2012) in the Swiss Alps. They observed that a rise of 10 ^{0}C in temperature increased the stem cycle amplitude of spruce and larch in 40%. Increased temperatures and day length combined, reduced the duration of the recovery phase (as seen in Figure 5.6) and increased the water lost by transpiration (Cermak *et al.* 2007). During the summer, the recovery phase was not sufficient to replenish the stem from the water lost during the day and trees had to resort to the internal water storage to keep up the transpiration demands (Zweifel *et al.* 2001; Sevanto *et al.* 2002; De Schepper and Steppe 2010).

The stem shrinkage observed during July (period 3) corresponded to the period when the ΔW presented a clear decreasing trend which can be associated with the exhaustion of the internal water storage. In the summer, soil water content diminishes and day length increases which decreases recovery. Ultimately the transpiration demands are not meet and the stem gradually contracts from one cycle to the next (Devine and Harrington 2011). Transpiration is controlled by stomatal responses to water availability (Jarvis and McNaughton 1986; Zweifel *et al.* 2006). To avoid drought-induced hydraulic failure, stomata closes during midday to maintain the water potential above the threshold of xylem cavitation (Loustau *et al.* 1996). The physiological consequences of stomata closure are carbon starvation and secondary growth decline, due to the allocation of carbon to higher ranking physiological processes such as root growth (Chaves *et al.* 2002; Oribe *et al.* 2003; Zweifel *et al.* 2006). As a result, trees reduce their metabolism and enter in quiescence (Cherubini *et al.* 2003; Makinën *et al.* 2008). In autumn, a period of re-hydration was observed in response to precipitation events. During the first 10 days of period 5, a series of cycles lasting more than 24 hours (long cycles) were observed, corresponding to a vigorous re-hydration.

The relationship between duration and amplitude of the stem radial variation phases changed during the year and reflected the prevailing climatic factors in each period. During period 3 the amplitude of recovery and increment was highly dependent on its duration. The recovery phase takes place mostly during the night. In the summer the days are longer which restricts the duration of recovery. If the duration of recovery is not sufficient to replenish the stem, then recovery will be limited and positive increments would not occur at all. As a consequence, the stem would progressively contract, which explains the negative increments observed during this period.

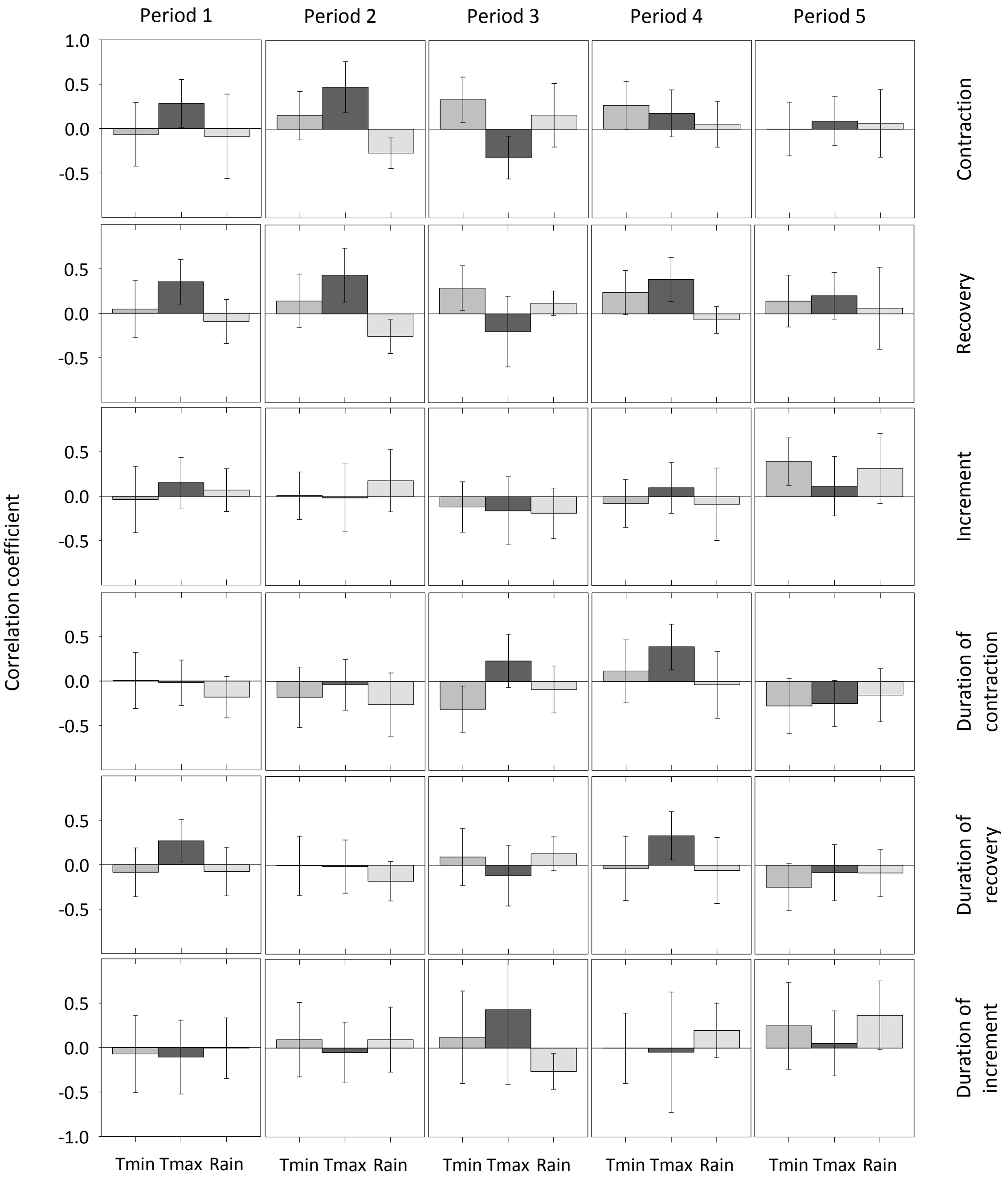

Figure 5.7 Bootstrapped correlations between amplitude and duration of each phase of stem radial variation and temperature and precipitation. The correlations are significant ($p<0.05$) when the confidence interval (twice the standard deviation, drawn as vertical error bar) is either higher or lower than zero.

During period 4 (summer), when tree water deficit was higher, a positive correlation was observed between the duration and amplitude of contraction. This relation is directly connected to the overall tree water status. During the summer the tree could not compensate for the water daily losses, presenting the most negative ΔW values. If the tree can no longer replenish the water lost by transpiration, then contraction would have to be restrained, which would result in a higher dependence between duration and amplitude. The opposite relationship was observed in periods 1 and 2, when the recovery phase was not limited, and the same amplitudes of contraction and recovery were achieved independently of duration. In autumn, the soil was replenished with water, thus amplitude and duration of recovery and increment were no longer dependent. However, due to the short duration of contraction during this period, a consequence of the long cycles, contraction amplitude was dependent on its duration.

5.4.2. Climatic response

The climatic response of duration was less clear than that of amplitude. During the first period, maximum temperature presented a positive correlation with recovery and contraction amplitudes and recovery duration, but no significant correlation with minimum temperature was observed. It is a well-documented fact that temperature can limit growth onset (Rossi *et al.* 2007; Deslauriers *et al.* 2008; Rossi *et al.* 2008). Rossi *et al.* (2008) determined that the average minimum temperature for xylogenesis was 4 – 5 °C. In the study site, the average minimum temperature in winter was 6 °C, which suggests that in the Mediterranean region, the minimum temperatures limiting cambial onset are higher. Several studies in the Mediterranean region have documented an earlier onset of cambial activity in response to higher winter temperatures (de Luis *et al.* 2007; Linares *et al.* 2009; Camarero *et al.* 2010). However, no threshold temperature has yet been determined for this area. The positive correlations observed in period 1 are indicative that trees were physiologically active during the winter. Corcuera *et al.* (2011) observed that, as in other Mediterranean evergreen conifers, maritime pine can maintain physiological activity all year round. It has also been demonstrated that cambial activity can be maintained during mild winters (Liphschitz and Levyadun 1986; Cherubini *et al.* 2003). In fact, maritime pine in the study area presented a long-lasting xylem differentiation persisting until December (*Chapter II*).

In period 2 the climatic conditions were optimal for tree growth with most of the increment being observed during this period. Previous dendrochronological studies on maritime pine showed that the climatic conditions observed in spring were the most determinant for tree-ring width (Vieira *et al.* 2009; Campelo *et al.* 2013). The climatic response of contraction and recovery was similar in this period, which, once again, demonstrates the interdependency of the two phases. The positive correlation found between contraction and maximum temperature suggests that transpiration rates were elevated. As temperatures rise, transpiration rates increase, promoting water loss and contraction (Zweifel *et al.* 2001; Cermak *et al.* 2007). Contraction and recovery also showed a negative correlation with precipitation in period 2. Studies on the diurnal course of transpiration showed that even irrigated trees experience stomatal closure if root water uptake is not sufficient to keep up with transpiration (White *et al.* 1996). The negative response of contraction to precipitation can be due to the indirect effect of clouds. Clouds would decrease direct solar radiance, thus decreasing leaf temperature, transpiration and ultimately contraction (King *et al.* 2012).

In July (period 3), the amplitude of contraction showed a negative correlation with maximum temperature. During this period, temperatures are high and soil water content is low, thus stomatal control on transpiration rates is stronger (Jarvis and McNaughton 1986; Zweifel *et al.* 2006). The stronger control of transpiration has a negative effect on contraction, reducing it. A positive correlation between amplitude of recovery and contraction and minimum temperature was also observed during this period. Lower temperatures benefit recovery, which translates in a better overnight hydration allowing a bigger contraction the following day.

In autumn (period 5), a positive correlation was observed between increment and minimum temperature. During this period there is a general decrease of temperature and photoperiod, which will induce a decrease in physiological activity, with the minimum temperature establishing the threshold for stem radius variation. Contraction did not show any correlation with the climatic parameters, revealing that expansion is a far more prominent phase during this period, as a consequence of the long cycles. Although stem re-hydration was observed during this period, no positive correlations with precipitation were registered. The re-hydration period, which corresponded to the long cycles, only lasted for 10 days whereas the rest of period 5 corresponded to the stabilization of stem size variation, and this may be the reason why the correlations were not significant.

5.5 Conclusions

The investigation confirmed the hypothesis that maritime pine stem radius variation has a daily and seasonal pattern that reflects the availability of water. Daily variations in stem radius were mainly determined by the course of transpiration and thus dependent on temperature and tree water status. The balance between water loss via transpiration and water uptake by the roots demonstrated a strong dependence between contraction and recovery phases.

Ultimately the rate of root water uptake and soil water content limited stem radius variation and the trees entered in a quiescent state, confirming the second hypothesis.

Temperature played an important role in stem size variation during the year, both directly by increased transpiration, and indirectly by decreasing soil water content via evapotranspiration. The increment onset of maritime pine was dependent on maximum temperature rather than minimum temperature, as observed on trees in cold environments. Nonetheless, water availability played the major role in stem radial variations. The severe water stress observed in summer caused the stem to contract and the tree to enter in a quiescent state, confirming the importance of water regulation in the survival of trees in a drought prone environment, such as the Mediterranean.

CHAPTER VI

General conclusions and future perspectives

6.1 General conclusions

Wood formation studies have received a great interest in the last years, with major findings regarding the physiology and climatic control of cambial activity (Deslauriers and Morin 2005; Gričar *et al.* 2005; Rossi *et al.* 2008; Seo *et al.* 2008; Rathgeber *et al.* 2011; Begum *et al.* 2013). However, most of these studies were held in temperate, boreal or altitude environments where temperature is the main factor limiting tree growth. It is thus important to expand these studies to other environments, namely drier ones, such as the Mediterranean. Cambial activity and wood formation have been occasionally studied in the Mediterranean region however this subject still remains poorly understood (de Luis *et al.* 2007; Linares *et al.* 2009; Camarero *et al.* 2010). The interaction and interchanging between external (e.g. temperature, water availability, and photoperiod) and internal factors (age, size and competition) controlling tree growth in the Mediterranean region make wood formation a very complex subject. In this dissertation the cambial activity and wood formation of maritime pine under Mediterranean climate were monitored in two years, 2010 and 2011, via anatomical observations and radial increment variation measurements, in order to meet the following objectives:

1. Determine the influence of timings and rates of cell production on xylogenesis;
2. Establish what causes the differences in diameter of trees with the same age;
3. Ascertain the influence of climate on cambial activity and xylem differentiation;
4. Define the daily and seasonal dynamics of stem radial variation and its relation to water availability;
5. And determine whether the bimodal pattern of growth, typical of the Mediterranean region, originates from a double reactivation of the cambium.

In **Chapter II**, trees of the same age, height, diameter at breast height and social status, but different tree-ring width in the past 15 years (1994-2009), were studied in order to determine what caused the differences in tree-ring width. It was observed that trees with larger tree-ring widths (fast growing trees), presented an earlier onset of cell enlargement and a faster growth rate in spring. However, the earlier start of cambial activity observed in the fast growing tress was not the responsible for the longer duration of xylogenesis but the higher rate of cell division, as described by Lupi *et al.* (2010) and Rossi *et al.* (2012). The higher rate of cell division lead to a larger accumulation of cells in the developing xylem and prolonged the differentiation phase, which delayed the end of wood formation. Thus, the differences if tree-ring width were due to rate, not timing of cell production (**Objective 1**).

In **Chapter III**, cambial activity and wood formation of trees with the same age but different diameters were compared. The onset of cambial activity was independent of tree size, supporting the previous findings that a common factor (e.g. temperature and/or photoperiod) was involved in the break of winter dormancy (**Chapters II**). This was contrary to the findings of Rathgeber *et al.* (2011), who found a strong relationship between the onset, end and duration of cambial activity and tree size. Although there were no differences in the timings of cambial onset between the diameter classes, enlargement and cell wall deposition lasted longer and presented a higher rate of cell production in large trees. The longer duration of xylogenesis observed in the large trees was probably due to a better access to resources, allowing higher rates of cell production (**Objective 2**).

Cambium was active from March to July, and quiescent from August to November, in both diameter classes. The minimum number of cambial cells was observed during the summer, suggesting a protection mechanism from hydraulic failure. A similar mechanism had been described in cold environments regarding freeze-induced cavitation (Zweifel and Hasler 2000; Turcotte *et al.* 2009). In November an increase in the number of cambial cells was observed, which was indicative of the break of summer quiescence. Fall precipitation re-hydrated the stem and brought the tree to a physiologically active state. Although cambial activity was observed in November, it was not followed by the differentiation of tracheids, demonstrating that maritime pine has the potential to form new xylem cells in autumn if the right climatic conditions are present. The annual periods of cambial dormancy and activity in the Mediterranean region are thus different from those proposed for temperate climates (Begum *et al.* 2013), and so should be the limiting factors.

In **Chapter IV**, the influence of climate on maritime pine cambial activity and xylem differentiation was monitored in two years (2010 and 2011). It was determined that the start of cambial activity was dependent on temperature, starting earlier in warmer years, which was in agreement with Deslauriers and Morin (2005) and (Rossi *et al.* 2008). However, the temperature threshold for cambial activity in the Mediterranean region was higher than the one reported for Boreal or altitude environments. The end of xylogenesis was associated to water stress, with an earlier stop observed in the drier year. It was also observed that a prolonged drought period had repercussions on the number and lumen area of latewood tracheids, with fewer cells with a smaller lumen area. Xylogenesis in the Mediterranean region appears to be under a double climatic control: temperature controls growth onset and water availability growth cessation (**Objective 3**).

In **Chapter V**, the daily cycles of stem radial variation in maritime pine were monitored during one growing season (2010), completing. The daily cycles of stem radial variation were studied in five periods of distinct physiological activity: winter dormancy, spring growth, pre-summer contraction, summer quiescence and autumn re-hydration. The amplitude and duration of the daily cycles changed during the year, in response to the climatic conditions (**Objective 4)**. Daily variations in stem radius were mainly determined by the course of transpiration and thus dependent on temperature and tree water status. The severe water stress observed in summer caused the stem to contract and the tree to enter in a quiescent period. In autumn, after the first rains, the stem re-hydrated rapidly restoring the tree to a physiologically active state.

The radial increment variation registered by band dendrometers proved to be useful to describe the seasonality of wood formation (**Chapters II**, **III** and **IV**). There was a delay between the onset of cambial activity observed in the anatomical study and the one recorded by the band dendrometers. This delay was due to the insufficient resolution of the band dendrometers to record the microscopic variations caused by the first increases in cambial cell number (Gruber *et al.* 2009). In both study years, the onset of radial increment corresponded to the start of the enlargement phase. Radial increment showed a clear bimodal pattern, with two periods of positive increment, in spring and autumn. However, although maritime pine has the ability to resume cambial activity after the summer drought (Vieira *et al.* 2009; Campelo *et al.* 2013), the second period of increment was mostly due to the re-hydration of the stem and to the differentiation of cambial derivatives, more likely produced by cambium during early summer (**Objective 5**).

Classical dendrochronological studies of maritime pine already provided indirect data on how tree-rings respond to climate (Vieira *et al.* 2009; Campelo *et al.* 2013). Monitoring wood formation throughout the growing season offered a method of direct observation on the climate-growth interaction, at a much more detailed time-resolution. It was determined that tree growth in the Mediterranean region was under a double climatic control, with growth onset in spring being limited by temperature, and the combined effect of high temperatures and low precipitation limiting tree growth in late spring/early summer. The relationship between climate and tree growth in the Mediterranean region is a complex one. Though the effect of a pronounced drought on maritime pine wood formation was successfully described, the response of cambium to other climatic scenarios needs further research.

6.2 Future perspectives

The results of the present thesis generated important information on the wood formation process of maritime pine growing in a drought-prone environment. However, further studies are necessary to improve the knowledge on the dynamics of cambial activity in the Mediterranean region. In order to fully understand the complex climate-growth relation, a long-term research would be necessary to include more years with different climatic characteristics.

To further understand wood growth dynamics under Mediterranean climate, aspects on water and carbon economy of trees should also be taken into account. Measurements of sap-flow would give important insights on the water economy of plants and measurements of photosynthesis and non-structural carbohydrates a perspective on carbon economy.

Another interesting direction would be to perform wood formation and physiological studies in trees of different provenances, in order to identify the populations that better adapt to drought conditions. The climate is changing, and it is predicted that the frequency and intensity of summer drought in the Mediterranean region will increase. Thus, the selection of drought resistance populations should be a top priority in future reforestations.

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"*Há um tempo em que é preciso abandonar as roupas usadas, que já tem a forma do nosso corpo, e esquecer os nossos caminhos, que nos levam sempre aos mesmos lugares. É o tempo da travessia: e, se não ousarmos fazê-la, teremos ficado, para sempre, à margem de nós mesmos*."

Fernando Pessoa