Cambial activity, wood formation and sapling survival of Pinus halepensis exposed to different irrigation regimes

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A R T I C L E   I N F O

Article history:
Received 21 May 2011
Received in revised form 8 July 2011
Accepted 8 July 2011
Available online 4 August 2011

Keywords:
Tree-ring
Cambium
Xylogenesis
Aleppo pine
Mediterranean
Intra-annual density fluctuations

A B S T R A C T

Wood formation and the anatomical characteristics of tree-rings play a decisive role in plant performance and survival due to the importance of water transport. Saplings may be especially vulnerable to water stress; however, the level of water stress that they can endure still remains largely unexamined. Thus, the general objective of our research is to determine the plasticity of wood formation in Pinus halepensis saplings. We evaluated cambial activity, xylem growth ring formation and its anatomical characteristics in different experimental irrigation conditions and tested for its influence on the success rate of sapling establishment. Our results demonstrate a high phenological plasticity in wood formation with juvenile P. halepensis saplings in relation to water availability. Dry conditions during spring and summer strongly limit their cambial activity, which is suggested to be linked to sapling survival during summer. Width and anatomical characteristics of tree-rings differ in saplings exposed to different irrigation conditions. Our results suggest that increasing water stress during spring and summer periods, as predicted by future climate change scenarios, may seriously affect the success of both natural regeneration and reforestation of P. halepensis, and this could severely modify potential distribution of the species.

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1. Introduction

The main characteristics of the Mediterranean climate are dry, hot and sunny summers, and mild, wet winters, with maximum rainfall in winter (Lionello et al., 2006). As a consequence, plants growing in the Mediterranean region have to survive relatively long periods of drought, and thus, the availability of water is considered to be the key factor driving eco-physiological processes and influencing photosynthetic rates, production of dry matter, allocation of carbohydrates to roots and the primary and secondary growth of woody plants (Cowling et al., 1996; Baquedano et al., 2008). Aleppo pine (Pinus halepensis Miller) is the most widely distributed pine in the Mediterranean Basin (Barbéro et al., 1998; Quézel, 2000) and the surface covered by the species has increased, due to plantation carried out during the 20th century and spontaneous colonization of abandoned land (Pausas et al., 2004). P. halepensis is described as the most drought-tolerant species of the genus Pinus (Barbéro et al., 1998; Zavala and Zea, 2004) and is, therefore, important for afforestation to protect soil in arid and semiarid conditions (Castillo et al., 1997), supply natural resources (Maestre and Cortina, 2004) and provide space for recreation (Schiller, 2001). The importance of P. halepensis may even increase in the future (Keller and Goldstein, 1998) since we expect longer and more severe drought periods (IPCC, 2007).

However, although P. halepensis adapts well to drought, the future of pine forest dynamics remains uncertain due to global warming. An increase in temperatures may give rise to an extended growing season (earlier onset and later cessation of activity) (Peñuelas et al., 2002) but this may not necessarily result in higher growth or survival rates, because a longer, more intense summer season can also be expected. Additionally, since recruitment plays a central role in plant population and community dynamics and summer drought is commonly found to be a major limiting factor both for seedlings and sapling survival, currently observed limitations on regeneration in Mediterranean forests may be exacerbated (Castro et al., 2004; de Luis et al., 2008; Mendoza et al., 2009; Uribeta et al., 2011).

Saplings can be especially vulnerable to water stress, due to their lower ability to take up resources from the soil (Savé et al., 1999; Lloret et al., 2004). However, although short-term effects of drought on growth and survival of trees of different species are well documented (Abe et al., 2003; Arend and Fromm, 2007; Giovannelli et al., 2007), the level of water stress that saplings can endure and the effect of this stress on survival and growth during the growing season still remains largely unexamined.
The study of cambial activity, xylem growth ring formation and anatomical characteristics of formed tree-rings, represents a promising approach in tree biology and climate change research (Fonti et al., 2010; Rossi et al., 2011). On one hand, intra-annual research into the radial growth of trees helps to elucidate how climate affects wood formation (e.g. Gribrated et al., 2006; Deslauriers et al., 2008; Cufar et al., 2008; Thibeault-Martel et al., 2008; Krause et al., 2003; Moser et al., 2010). On the other hand, it enables us to determine how climate variability may modify both growth rates and the anatomical characteristics of formed tree-rings playing a decisive role in defining hydraulic and mechanical properties of wood (Froux et al., 2002; de Micco et al., 2008; Martinez-Meier et al., 2008; Hoffmann et al., 2011).

Since cambial activity, wood formation and anatomical characteristics of formed tree-rings play a decisive role in plant performance and survival (Lipschitz and Lev-Yadun, 1986; Rossi et al., 2009a), the availability of this information may be critically important in evaluating the range of plasticity in species under different environmental conditions as a first step in predicting their responses to future climatic scenarios.

Research into xylogenesis requires direct observation of cambium and xylem tissues during the growing season and, therefore, the current state of knowledge is limited to short-term periods (see Rossi et al., 2011 as exception), and how trees respond to a wider range of variability in main climate conditions remains virtually unexplored. All this suggests that controlled experiments are needed to improve our knowledge on how the main limiting factors affect cambial activity and wood formation (Gribrated et al., 2006; Rossi et al., 2009b; Eilmann et al., 2010; Fonti et al., 2010; Jyske et al., 2010; Moser et al., 2010).

However, despite its relevance, the intraspecific plasticity of xylem phenology has been largely overlooked in Mediterranean areas (see de Luis et al., 2007; Battipaglia et al., 2010; Camarero et al., 2010; or de Luis et al., 2011 as recent exceptions). Moreover, of the studies that do exist, most are based on adult trees and the conclusions obtained cannot be assumed to be valid for juvenile saplings.

Thus, the general objective of our research is to determine the extent of plasticity of wood formation in P. halepensis saplings. To this end, we used an experimental approach to evaluate cambial activity, xylem growth ring formation and its anatomical characteristics, and sapling survival in different experimental irrigation conditions.

On the basis that, in semiarid conditions, water availability represents the key environmental factor promoting cambial activity and wood formation (de Luis et al., 2007; Camarero et al., 2010; de Luis et al., 2009, 2011) we hypothesized that under continuous irrigation conditions, cambial activity and cell differentiation can be maintained throughout the whole year. Contrarily, under natural (non-irrigated) conditions, we expect that summer drought will result in decreased cambial activity, but will increase in autumn if irrigation occurs.

We also expect that, since water transport strongly depends on newly formed xylem cells, sapling mortality during summer drought will depend on cambial production and differentiation of xylem cells in spring and early summer. In addition width and anatomical characteristics of tree-rings will differ in saplings exposed to different irrigation conditions.

2. Materials and methods

2.1. Study site

The study was carried out in sixteen plots in the forest nursery of Guardamar del Segura on the south-east coast of Spain (38°48′N, 0°40′W; 5 m a.s.l.). Mean annual temperature is 18.0 °C and mean annual precipitation is 309 mm (de Luis et al., 2010; González-Hidalgo et al., 2011). During the experimental year 2005, the mean annual temperature was 1 °C higher (19 °C) and annual precipitation 28% lower (223 mm) than the long term averages (Fig. 1).

2.2. Experimental design

The experiment involved 512 3-year-old saplings of P. halepensis grown in a glasshouse under continuous irrigation until mid-October 2004. The saplings were then planted outside in sixteen experimental plots, in a completely randomized design. When planted, the saplings were uniform in height (25.3 ± 3.2 cm) and diameter (2.1 ± 0.3 cm).

On each of the experimental plots (3 x 18 meters) were planted 32 randomly selected saplings. The distance between them was 1 m. A drip irrigation system supplied water to all plants from the date of planting until the end of February 2005, when different experimental treatments were applied.

The treatments consisted of plots irrigated seasonally (10 mm daily) as to maintain no water stress (irrigated plants) and plots in which no water was supplied (control plants). Experimental design involved 2 plots per treatment. An overview of experimental conditions is presented in Table 1. Thus, from the original 16 experimental plots, 2 plots were continuously irrigated during spring (March, April and May), summer (June, July and August) and autumn (September, October and November). Another 2 plots were subjected to natural conditions (no water was supplied) during the same period (control plots) and the remaining 12 plots were subjected to a combination of seasonal irrigation and natural conditions.

2.3. Sampling procedure and sample preparation

Sapling survival was monitored monthly from March to November 2005. In addition, tree-ring formation was studied at monthly intervals by collecting the stem disks 2 cm above the root collar, always from 4 randomly selected living plants from each treatment. Thus, at the end of March, April and May 2005, four irrigated (R) and four non-irrigated (C) saplings were extracted and analyzed. At the end of summer months, 4 additional saplings were randomly selected and extracted from each experimental treatment (4 saplings from R plots, 4 from RC; 4 from CR and 4 from CC).
From September to end of November, we extracted 4 additional saplings from each experimental treatment each month (4 RRR; 4 RRC; 4 RCR; 4 RCC; 4 CRR; 4 CRC; 4 CCR and 4 CCC saplings).

Immediately after removal from a sapling, stem disks were fixed in FEA (formalin-ethanol-acetic acid solution) and afterwards dehydrated in a graded series of ethanol (30%, 50%, and 70%). Transverse sections of stem disks (25 µm thick) were prepared with a Leica SM 2000R sliding microtome. Finally, all sections were double-stained, first with Safranin (0.5% in 95% ethanol) and then with Astra blue (0.5% in 95% ethanol), and embedded in Euparal resin. The equipment used for observations and semi-automatic counting consisted of: (a) a Nikon Eclipse 800 light microscope (bright field and polarized light), (b) digital camera DS-Fi1, and (c) NIS elements BR3 image analysis system.

We followed the seasonal dynamics of xylem formation by distinguishing among cambial cells, differentiating tracheids [expansion, secondary cell wall formation and lignification] and mature tracheids (Fig. 2). The cambium consisted of radially flattened cells with thin, non-lignified primary cell walls that stained blue with astra-blue. Expanding cells were defined by larger radial dimensions and thin, non-lignified, blue-stained cell walls. Deposition of secondary wall layers was observed under polarized light as the cell walls showed birefringence and the thickness of the originally thin cell walls started to increase. The beginning of cell wall lignification could be observed as the red staining by safranin gradually replaced the blue staining, progressing from the outer parts of the cell wall towards the lumen. When the process of differentiation was completed, the cell walls of mature tracheids were completely red stained and the cell lumina were empty (Fig. 2).

From December 2005 to June 2006, the remaining saplings in the plots continued to grow under natural conditions without irrigation. At this point, all saplings were extracted and a stem disk taken from each one. The disks were dried and polished to study the ring-width and the anatomical characteristics of the tree-rings formed in 2005. Thus, the widths of the tree-rings formed during 2005 were measured to the nearest 0.01 mm using TSAP Win program. Furthermore, tree-rings were examined for intra-annual density fluctuations (IADFs) with a stereomicroscope, magnification 4–100×. The rings with typical earlywood and latewood and with a more or less gradual transition among them were defined as normal rings (Fig. 3a). IADFs were identified when this gradual

### Table 1

<table>
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<tr>
<th>Treatment codes</th>
<th>SPRING</th>
<th>SUMMER</th>
<th>AUTUMN</th>
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<tr>
<td></td>
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<td>Apr</td>
<td>May</td>
</tr>
<tr>
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<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>RRC</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
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<td>Yes</td>
<td>Yes</td>
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<td></td>
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<tr>
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<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>CCC</td>
<td></td>
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</table>

Fig. 2. Cambium activity during 2005 growth season. (a) Increased number of cambial (CC) and expanding cells (PC) give evidence that cell divisions on 25 February already started. (b) Period of maximal cell production with xylem cells in different phases of cell development; PC, secondary wall formation (SW) and mature cells (MT) (2 June). (c) Dormant CC on and mature xylem below it on 26 December. Scale bar 100 µm.

Fig. 3. Structure of different types of tree-rings formed in 2005 in *Pinus halepensis*. (a) Normal growth ring. (b) E-ring with latewood like cells in earlywood (arrowhead). (c) L-ring with earlywood like cells in latewood (arrowhead). Dashed lines represent the boundary between 2004 and 2005 tree-rings. Scale bars 100 µm.
transition was interrupted. They were classified according to latewood-like cells in earlywood (E-ring; Fig. 3b) and earlywood-like cells in latewood (L-ring; Fig. 3c) (Campelo et al., 2007; de Luis et al., 2011).

2.4. Statistical analysis

Since the data distribution within each sampling date did not follow the assumption of normality, the differences in the number of cambial cells observed from March to April 2005 during the seasonal irrigation experiment with different treatments. Error bars represent the 25th and the 75th percentiles within each experimental block. Bold letters indicate seasonal irrigation treatment. C: Control not irrigated, R: irrigated. Gray areas indicate the envelope from all experimental units.
of cambial cells and differentiating tracheids among the treatments were analyzed for each sampling date by using the nonparametric Kruskal–Wallis $H$-test.

In contrast, because there was no violation of the assumption of normality, differences among the treatments in regard to final ring-widths were analyzed by one-way ANOVA, with final irrigation treatments as main factors. Post-hoc comparison of the means was made using the Bonferroni test.

### 3. Results

#### 3.1. Cambial activity

The state of the cambium and consequently the mean number of cambial cells in *Pinus halepensis* saplings varied in the growing season of 2005, and also in regard to experimental conditions. The cambium was mostly in a dormant state when the number of cells was around 5 layers or less. During the period of cell divisions, the number of cambial cells increased and expanding xylem cells adjacent to the cambium were clearly visible.

Temporal variation in the number of cambial cells in each irrigation treatment is shown in Fig. 4. Lower mean values (4.5 cells), indicating no cambial activity, were observed in May in non-irrigated plants (Fig. 4g and h) and in June in plants irrigated during spring but with dry conditions during June (Fig. 4c). However, higher mean values (8.25 cells) and actively dividing cambial cells were observed in October in saplings that were under irrigation during spring, summer and autumn period (Fig. 4a).

Irrigation treatments have a significant and positive effect on the number of cambial cells in *P. halepensis* saplings (Table 2 shows Kruskal–Wallis statistics and significance levels).

Thus, we observed that the number of cambial cells increased due to irrigation during spring (March–May). This effect is already significant in March (1 month after the beginning of irrigation treatment) and also in April and May. However, we also noticed that irrigation in spring did not have any significant long term effect on cambial cell production, since the number of cambial cells in subsequent months did not differ significantly from the spring treatment (Table 2).

Similarly, cambial cell production in *P. halepensis* saplings increased due to irrigation during the summer period (June–August). This means that a significantly higher number of cambial cells were found in plants irrigated in June, July and August. However, unlike the spring treatment, the positive effect of irrigation on cambial production in the summer is still significant at the beginning of autumn; in September (Table 2).

The effect of irrigation during autumn (September–November) did not have an immediate effect on cambial cell production. The number of cambial cells did not significantly differ between plants with or without irrigation at the end of September. However, the effect of autumn irrigation is evident in October, and again no effects are observed in November (Table 2).

### 3.2. Wood formation

Newly formed xylem cells are a result of cambial activity. The first stage of differentiation is cell expansion; therefore, we counted the number of expanding tracheids to track the dynamics of xylem growth ring formation. The mean values of expanding cells observed in *P. halepensis* saplings were closely related to cambial activity. The number of expanding xylem cells differed over time and experimental conditions (Fig. 5).

The number of expanding cells followed a clear bimodal pattern in saplings exposed to continuous irrigation during spring, summer and autumn (RRR treatment). The mean number of expanding cells is 3.5 layers in March; it decreased to zero in July and afterwards increased again to 4 cell layers in October (Fig. 5a).

A slow decrease in the number of expanding cells during summer was observed in saplings under continuous irrigation during spring and summer (RRC treatment, Fig. 5b). A different pattern of radial growth is perceived in the saplings growing in dry conditions during spring, but irrigated during summer (CR treatments) (Fig. 5e and f). More specifically, the number of expanding tracheids decreased from March (around 2.5 cell layers) to May (no cells), but in the summer months, the number of cells slowly increased to reach maximum in autumn (September–October).

On the other hand, the number of newly formed xylem cells in the saplings without irrigation during the entire growing season (CCC treatment) rapidly decreased during the spring months. We detected almost no newly formed xylem cells in summer and no clear cambial reactivation in autumn, despite above average precipitation in September (Fig. 5h).

Irrigation had a positive influence on the number of cambial cells and higher cell production, and this was reflected in an increased number of expanding cells (Table 3). The effect of spring irrigation was not significant in March, but was observed in April and May. Summer irrigation gave rise to a higher number of expanding tracheids in summer (from June till August), also in September. Irrigation in autumn had a positive effect on the number of expanding cells in October (Table 3).

### 3.3. Sapling survival

The experimental plots returned the highest mortality rate of saplings during summer (July and mainly August), but there were differences among treatments. All saplings survived on plots irrigated during spring and summer (RR’ and RC’ treatments). On the contrary, mortality was higher (almost 80% of saplings) on plots without a water supply during spring and summer (CC’ treatments). Approximately 20% mortality was observed in saplings in plots with no water supply during spring, but with irrigation in summer (CR’ treatments) (Fig. 6).

### 3.4. Characteristics of xylem growth rings

As a consequence of the differences in the dynamics of cambial activity and wood formation described above, the final width of

### Table 2

Effect of seasonal irrigation treatments (SPRING, SUMMER and AUTUMN) on the number of cambial cells in *Pinus halepensis* saplings in different months of the growing season (from March to November). Kruskall–Wallis statistics and significance levels ($p$). Significant values at $p < 0.05$ are bold.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mar</th>
<th>Apr</th>
<th>May</th>
<th>Jun</th>
<th>Jul</th>
<th>Aug</th>
<th>Sep</th>
<th>Oct</th>
<th>Nov</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spring</td>
<td></td>
<td></td>
<td></td>
<td>5.46</td>
<td>4.05</td>
<td>5.67</td>
<td>0.05</td>
<td>0.00</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.05</td>
<td>0.00</td>
<td>0.99</td>
<td>0.25</td>
<td>0.49</td>
<td>0.05</td>
</tr>
<tr>
<td>Summer</td>
<td></td>
<td></td>
<td></td>
<td>0.824</td>
<td>1.000</td>
<td>0.321</td>
<td>0.615</td>
<td>0.485</td>
<td>0.820</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>8.33</td>
<td>4.05</td>
<td>5.67</td>
<td>0.05</td>
<td>0.00</td>
<td>0.99</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.04</td>
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<td>0.735</td>
<td>0.352</td>
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<td>Autumn</td>
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<td></td>
<td></td>
<td>0.86</td>
<td>14.04</td>
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xylem growth rings in *P. halepensis* saplings differed significantly according to the treatments. Hence, the ring-width was significantly higher in saplings exposed to continuous irrigation (RRR treatment), whereas lower values were observed in saplings growing in dry experimental conditions during spring and autumn (one-way ANOVA: $F = 42.1$; d.f. = 7, 99; $p > 0.001$) (Fig. 7).

The anatomy of wood in saplings exposed to different conditions also varied. We detected different patterns of intra-annual density fluctuations (IADF), with E-rings being found in more than
50% of the analyzed saplings exposed to dry conditions in spring and irrigated in summer (Fig. 8 a). On the other hand, L-rings mainly occurred in the saplings growing under continuous irrigation treatments (Fig. 8 b).

### 4. Discussion

Until a short time ago, most of the data on radial growth of trees were obtained from dendrochronological studies. However, although the tree-ring chronologies provide information on interannual to multi-centennial time scales it was only recently made clear exactly when ring formation takes place, and how specific environmental factors influence the rate of cell division in the cambium and cell differentiation that collectively determine tree-ring characteristics (Camarero et al., 2010; Moser et al., 2010). Studies on wood formation and experiments in controlled environments have contributed to answering these questions (see review of Fonti et al., 2010).

In this respect, *P. halepensis* is a relatively well investigated tree species and different studies on adult trees highlighted plasticity on the seasonal dynamics of cambial growth in dependence on climate conditions (Gindel, 1967; Serre, 1976a,b; Liphschitz et al., 1984; Attolini et al., 1990; Nicault et al., 2001). In this line, results from our irrigation experiments demonstrate that, contrary to observations in saplings in cold environments (Rossi et al., 2009a), the duration of the growing season in *P. halepensis* saplings growing in dry, warm, semiarid conditions can be 9 months or more.

Moreover, our experiment suggests that bimodal patterns of cambial activity and cell differentiation could be genetically defined in *P. halepensis* saplings since, even under continuous irrigation, summer represents a period when cambial activity and wood...
formation slow down. However, such a bimodal pattern could be anticipated or delayed in dependence on climatic conditions and possibly the state of the cambium in spring. Thus, cambial activity and cell differentiation under wet (irrigated) summer conditions only occur if spring is dry (no irrigation) while if the amount of water in spring was sufficient for cambial activity, cell division stopped or slowed down in summer, even if the trees were irrigated. Therefore, our experiment confirms our hypothesis that *P. halepensis* saplings are very plastic being quite able to adapt its radial growth rhythm to the availability of water.

In addition, if water is available during summer, saplings can resume cambial activity and wood formation in autumn, which is seen as a bimodal pattern of activity. As expected, cambial reactivation was related to the availability of water in autumn. However, the intensity of reactivation also depends on conditions earlier in the growing season (spring and summer).

Our results also indicate that sapling mortality during dry summer conditions is significantly related with dry conditions in spring and early summer that promote the lack of cambial production and differentiation of xylem cells. Several studies have demonstrated that xyllogenesis is an age-dependent process and that it differs in juvenile and adult trees in terms of dynamics and timing (Rossi et al., 2008, 2011). However, our results from juvenile saplings show some similarities with those observed in adult trees. Thus, we found that cambial activity can take place for 9 months or more in juvenile saplings, and this duration was also observed in adult *P. halepensis* trees growing in semiarid conditions (de Luis et al., 2007, 2011). Similarly, in adult trees, cambial activity can be more or less continuous or with one or two interruptions defined by climatic conditions (de Luis et al., 2007, 2011; Camarero et al., 2010). However, unlike adult trees, our results suggest that cambial activity during spring and early summer may be critically important to the survival of saplings, especially in the first part of the growing season after planting, when the root system has not yet developed and newly formed wood is crucial for providing enough conductive tissue for water transport. In adult trees, older tree-rings in the sapwood preserve the ability to conduct water; therefore, partial or complete absence of xylem formation in one or a few years does not affect tree survival (Zimmermann, 1983; Pallardy, 2008). Thus, adult trees can survive even though wood (on the stem) is not formed every year (Raventós et al., 2001; Cherubini et al., 2003; Novak et al., 2011).

Our findings in juvenile saplings suggest also that different irrigation conditions affect both the widths and the anatomical characteristics of tree-rings. In adult trees, if early spring conditions are abnormally warm but late spring become wetter, E-rings are formed (Campelo et al., 2007; de Luis et al., 2011). Contra- rily, our results in juvenile saplings suggest that E-rings were formed if it was dry in spring and wet in summer. Furthermore, in adult trees, E-rings are formed if summer is wet, but in juvenile saplings similar climate conditions promote L-ring only in saplings with very productive cambium. Similar differences between juvenile saplings and adult trees have also been found in other tree species from several environments with a different length of growing season (de Micco et al., 2008; Battipaglia et al., 2010).

The influence of ring-width and wood anatomy on future sapling growth and survival was not the aim of our study. However, earlywood and latewood cells have very different hydraulic and mechanical properties. Earlywood cells in conifers are more important for water transport, whereas latewood cells provide mechanical stability (Froux et al., 2002). Therefore, any changes in the proportion of earlywood and latewood could very likely modify the hydraulic and mechanical properties of wood and such changes might affect future sapling development (de Micco et al., 2008; Martinez-Meier et al., 2008; Hoffmann et al., 2011).

5. Conclusions

Within the perspective of global warming, tree survival is certainly related to their ability to preserve their hydraulic system, and the functional phenological plasticity of wood formation has been commonly described as an important adaptation of trees to new, changed climate conditions.

Our results indicate that such plasticity exists in *P. halepensis* saplings. Dry conditions during spring and summer strongly limit their cambial activity, which is suggested to be linked to sapling survival during summer. In this respect, saplings may be especially vulnerable to a predicted increase in water stress during spring and summer, as it may limit their ability to survive. This could affect both natural regeneration and the success of reforestation, leading to a change in the distribution of the species in the future.

Acknowledgments

This study was supported by Spanish Ministry of Education and Science co-funded by FEDER Program (Project: CGL2008-05112-C02-01) and by the Slovenian Research Agency (Programme P4-0015 and P4-0107 and the Young Researchers’ Programme). We thank Elaine Rowe for her work in improving the English of this manuscript.

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