

Intra-annual patterns of tracheid size in the Mediterranean tree *Juniperus thurifera* as an indicator of seasonal water stress

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1 **ABSTRACT**

2 Since climate can affect xylem cell anatomy, series of intra-annual cell anatomical
3 features have the potential to retrospectively supply seasonal climatic information. In this
4 study we explored the ability to extract information about water stress conditions from
5 tracheid features of the Mediterranean conifer *Juniperus thurifera*. Tracheidograms of four
6 climatic years from two drought-sensitive sites in Spain were compared to evaluate whether it
7 is possible to link intra-annual cell size series to seasonal climatic conditions.

8 Results indicated site-specific anatomical adjustment, such as smaller and thicker
9 tracheids at the dryer site, but also showed a strong climatic imprint on the intra-annual
10 pattern of tracheid size. Site differences in cell size reflected expected structural adjustments
11 against cavitation failures. Differences between intra-annual patterns, however, indicated a
12 response to seasonal changes in water availability, whereby cells formed under drought
13 conditions were smaller and thicker, and vice versa. This relationship was more manifest and
14 stable at the dryer site.

15

16 **Keywords:** drought, tgram R-package, tracheidogram, tree rings, wood anatomy.

17 **INTRODUCTION**

18 The wood anatomical structure of woody plants is mainly genetically determined and
 19 can therefore be used as a trait for taxonomic classification. However, there is a certain intra-
 20 specific variability that is subject to environmental modification (Wimmer 2002,
 21 Schweingruber 2007). In fact trees have to adjust their xylem structure in order to fit the
 22 ecological settings of their habitat and the seasonal and inter-annual climate variability (Fonti
 23 et al. 2010). By performing these adjustments, the environmental conditions that occur during
 24 wood formation – i.e., the conditions affecting processes of cell division, differentiation,
 25 enlargement and wall thickening – leave a permanent mark on cell anatomical characteristics
 26 (Denne and Dodd 1981) and thus, like other tree-ring proxies such as ring width or maximum
 27 latewood density, they can be assigned to a calendar year and used to indirectly reconstruct
 28 past environmental dynamics (Fritts 2001).

29 Recent analyses of inter-annual variability in the size of water-conducting cells –
 30 vessels in deciduous trees and tracheids in conifers – have demonstrated that these cells can
 31 carry sub-annual information that reflects changes in environmental conditions (see Fonti et
 32 al. 2010 for a review). Temperature has been observed to mainly affect physiological
 33 processes involved in wood formation as synthesis and transport of hormones (e.g., Creber
 34 and Chaloner 1984, Uggla et al. 1998, 2001, Oribe et al. 2003). So for example, it has been
 35 observed that previous fall and spring temperatures can affect earlywood vessel size of the
 36 ring-porous *Castanea sativa* (Fonti et al. 2007) and that tracheid size (lumen diameter and
 37 wall thickness) in *Picea mariana* and *Larix cajanderi* is positively correlated with summer
 38 temperature (Wang et al. 2002, Panyushkina et al. 2003). In contrast, water availability seems
 39 to principally influence cell turgidity during cell expansion (e.g., Vysotskaya and Vaganov
 40 1989, Gindl 2001). Experiments performed on tree saplings have indicated that water deficit
 41 reduces cell number and tracheid lumen diameter, but increases cell wall thickness (Sheriff
 42 and Whitehead 1984, Abe and Nakai 1999, Abe et al. 2003, Rossi et al. 2009, Gallé et al. in

press). Coherent changes in cell structure have also been observed along ecological gradients. Cell numbers, latewood radial cell diameter, cell wall thickness and wood density have been observed to decrease with increasing latitude (Wang et al. 2002, St-Germain and Krause 2008) and latewood density to decrease with elevation (Splechtna et al. 2000).

Since trees growing in Mediterranean continental climates are subjected to limitation from both climatic factors, namely cold temperatures in winter and drought stress in summer (Terradas and Savé 1992, García-Plazaola et al. 1997), it might be interesting to investigate whether their anatomical structure is reflecting the environmental limiting conditions. *Juniperus thurifera* is a continental Mediterranean endemic conifer species forming distinct annual rings with very thin latewood (DeSoto 2010). This species can be found in both the more cool and humid northwest and central parts of Spain, as well as in the warm, dry regions of the eastern Iberian Peninsula (Amaral-Franco 1986). Previous dendroclimatic studies in central Spain and the French Pyrenees have shown that secondary growth of *J. thurifera* is mainly controlled by winter precipitation, spring temperature and summer water stress (Bertaudière et al. 1999, Rozas et al. 2009).

In this study we compared two groups of Mediterranean *J. thurifera* trees growing under contrasting water conditions with the aim i) to recognize intra-annual wood cell anatomical patterns, ii) to assess whether ring width and cell anatomical responses differ between regional climates and, iii) to verify if these differences can be used to link intra-annual patterns of cell anatomical features to seasonal climatic conditions.

66 MATERIAL AND METHODS

67 Study site, sampled trees and tree-ring analyses

68 Wood samples were collected from dominant, healthy and mature *J. thurifera* trees
 69 growing at two sites located at the eastern and western boundaries of the species distribution
 70 area (Fig. 1). Located in northwest Spain near Palencia, the Peña Lampa site (LA; 42° 50' 31"
 71 N, 4° 51' 36" W; 1187 m a.s.l.) encompasses the coolest and most humid region of its
 72 distribution. In contrast, the Santa Engracia site (SA; 41° 46' 35" N, 0° 32' 29" W; 530 m a.s.l.)
 73 near Zaragoza in northeast Spain is located in the warmest, driest part of the distribution
 74 range. The sites are about 350 km apart. Both site's soils are calcium rich.

75 In total 40 trees were sampled, 20 in LA and 20 in SA. Sizes of the trees sampled
 76 between sites are comparable. Trees in LA were on average 6.1 m height (standard deviation
 77 of ± 1.5) and showed a diameter breast height (DBH) of 20.7 cm (± 6.2). SA trees averaged
 78 5.7 m in height (± 0.6) and 18.9 cm in DBH (± 3.7). Trees in LA were on average 95 years
 79 old and (± 18.8), and SA trees averaged 77 years (± 20.9).

80 Two 5-mm-diameter cores were extracted from each tree at stem breast height and
 81 perpendicular to the slope direction. Cores were glued on wooden mounts and polished for
 82 ring width measurement. Tree rings were measured to a resolution of 0.001 mm using a
 83 Velmex sliding-stage micrometer (Bloomfield, NY, USA.) and visual cross-dating was
 84 verified using COFECHA (Grissino-Mayer 2001).

85 Climate–growth relationships were established by computing Pearson's correlations
 86 between the ring-width site chronologies and the monthly average temperature and total
 87 monthly precipitation. The chronologies were built by averaging the age detrended time-
 88 series. The age trend was removed by first applying a negative exponential function and then
 89 a 64-year cubic smoothing spline. Climatic data were obtained for the 0.5° grids of the TS 3.0
 90 dataset (CRU 2008) closest to the tree sites for the period from 1961 to 2002 (42 years).

91

92 **Wood anatomical measurements**

93 The analysis of tracheid anatomical measurements has been performed on four
 94 specific annual rings (1979, 1986, 1991, 1996) from a set of tree cores whose ring-width
 95 series were highly inter-correlated with the mean chronology of the corresponding site ($r >$
 96 0.5). We selected these four years on the basis of their climatic conditions: 1979 and 1996
 97 displayed a precipitation maximum in winter, 1986 and 1991 a precipitation minimum in
 98 summer. In total, 13 series from 8 trees for LA and 10 series from 6 trees for SA were
 99 selected, for a total of 92 annual rings. Comparisons of age and size of selected trees between
 100 the sites were not significantly different (age: $t_{12} = 0.34$; diameter: $t_{12} = -1.55$; height: $t_{12} =$
 101 1.56; $P > 0.05$) and sexes were equally distributed within the sites in order to control the
 102 individual effects.

103 To perform cell anatomical surveys using digital images of the wood core cross-
 104 section, the core surface was prepared using a core microtome (Gärtner and Nievergelt 2010)
 105 The contrast between the tracheid lumina and the cell wall was improved by staining the
 106 wood surface with black printer ink (Canon Cartridge BCI-6BK, Canon Inc., Japan). Images
 107 of ring cross-sections (RGB, color 24 bit) were captured using a digital video camera
 108 (ColorView III, Soft Imaging System, Germany) connected to a transmitted light microscope
 109 (Olympus BX41, Japan) with a 20x objective (image resolution: $0.17\mu\text{m}/\text{pixel}$). Since the
 110 field of view through the objective was not large enough to cover the entire annual ring width,
 111 adjacent images from the same annual ring were merged using the photo stitching program
 112 PTgui. An example of a captured image is shown in Figure 2a.

113 For each selected ring, the radial lumen diameter (LD) and the radial cell wall
 114 thickness (CWT) were measured along five tracheid files using the “plot profile” function of
 115 the image analysis program Image J (version 1.40g; Abramoff et al. 2004), which was used to
 116 discriminate between tracheid cell walls and cell lumina according to the variation of the grey
 117 value along a line passing through a tracheid radial file (Fig. 2b). Tracheid files showing the

tangentially largest cells were selected for measurement in order to consider only tracheids which were cut close to the middle of their longitudinal length. The radial tracheid diameter (TD) and the ratio LD/CWT were then calculated (Fig. 2c). Cell anatomical data per file were transformed in a tracheidogram – i.e., a curve showing variations in cell parameters as a function of the cell position within an annual ring – using the procedure described by Vaganov (1990). This method normalizes the varying number of cells between radial files and annual rings to a fixed value. Tracheidograms were based on a normalized number of 23 cells (20 earlywood and 3 latewood) per ring. Latewood tracheids were identified according to the Mork index (Denne 1988), i.e., when cell lumen is smaller than twice the cell wall. We have included a set of R functions to compute LD, CWT, TD and the ratio LD/CWT from the grey value line and the function to compute normalized tracheidograms in the new R-package "tgram" (available from CRAN; <http://cran.r-project.org>; Fig. 2)

Calculation of water stress and its relationship to tracheid anatomy

Daily water balance (WB) over the year was estimated as the difference between the accumulated daily precipitation starting on January 15th and measured at the closest climatic stations of Velilla del Rio Carrión (LA) and Zaragoza Aula Dei (SA) provided by AEMet (Agencia Estatal de Meteorología) and the potential evapotranspiration estimated for each site as a function of the mean monthly temperatures and the geographical latitude (according to Thornthwaite 1948). Average water balances over 10-day periods from March 1st to November 30th (27 data points) have been linked to the deviation of the observed tracheid diameter from the average tracheidogram of the four selected years of each site, which is considered an estimate of the site specific “normal pattern” for cell size variation over the season. The average tracheidogram has been fitted using a fourth order polynomial function. Deviation was calculated as the difference between the observed tracheid diameter and the fitted function.

Statistical analysis

Generalized estimating equations (GEEs) were used to test the effect of years and sites on the wood anatomical features. GEEs are an extension of generalized linear models (GLMs) to model correlated data (Quinn and Keough 2002). Models included the year, sex and site as fixed factors and their interactions. We assumed a normal error distribution with an identity link function for mean LD, TD and CWT and also for logarithmic transformation of earlywood, latewood and total ring width. A Poisson error distribution with a log link function was assumed for the number of tracheids and for the latewood percentage. Tracheidogram parameters were analysed using multi-level models (MLMs) that fit linear models for longitudinal data over time (Omar et al. 1999, Quené and van der Bergh 2004). Models included year, site and position as explanatory fixed factors and all the possible two-way interactions among them. We considered the anatomical features LD, CWT, TD and LD/CWT ratio as response variables and assumed a normal error distribution with an identity link function for all of them. Least-square means differences were tested pairwise, by using the DIFF option in the LSMEANS statement for both models. We fitted GLMs and MLMs via restricted maximum-likelihood (REML) using the GEMMOD and MIXED procedures of SAS, respectively (SAS Statistical package 9.1).

RESULTS

Climatic conditions and water stress during the selected years

Due to the higher yearly average temperature (14.7 °C) and lower precipitation sum (309 mm), the trees at the SA site suffered from longer and more intense periods of water stress than trees at the LA site (9.0 °C and 844 mm; Fig. 1). At SA, the water stress period, i.e. the period with negative water balance, reached a maximum of -121 mm in June and lasted two months longer (April to September) than at LA (-71 mm, June to September).

For the selected climatic years, the years 1979 in LA and 1996 in SA were characterized by extremely wet winters (+130% and 113% in January to March) followed by

a relatively dry spring and summer in comparison to the long-term average (1961–2002). In contrast, 1986 and 1991 were characterized by a dry spring and summer, especially at LA (Table 1). Climate parameters were calculated over the period 1961–2002 obtained from the CRU dataset of the corresponding 0.5° grid.

Tree-ring growth and response to climate

Trees at the dryer and warmer SA site showed larger ring widths (average 1961–2002 is 0.86 mm vs. 0.59 mm) and higher mean sensitivity ($ms_x = 0.32$ vs. 0.22) than those at the wetter and cooler LA site, but both sites had similar common signals ($r_{bt} = 0.30$ vs. 0.32; Fig. 3a). Climate-growth relationships indicated that trees at both sites responded similarly to summer conditions, with a negative response to mean temperature (July $r = -0.35$ in SA vs. June $r = -0.35$ in LA; $P < 0.05$) and a positive response to total precipitation (July $r = 0.30$ in SA vs. aggregate May-June-July $r = 0.42$ in LA; $P < 0.05$). In contrast, the climatic response to winter precipitation clearly differed between sites. The radial growth of trees at SA responded positively to precipitation (December-January $r = 0.35$; $P < 0.05$), whereas growth of LA trees responded negatively (November to March $r = -0.41$; $P < 0.05$; Fig. 3b).

Differences in cell numbers and sizes

The ring widths of the analyzed years ranged from a minimum of 275 μm (15 tracheids, 1991 in LA) to a maximum of 500 μm (27 tracheids, 1979 in SA; Table 2). On average, 9 to 14% of xylem cells in the tree rings were classified as latewood. In the earlywood, the mean number of tracheids per radial file was lower at LA (12.86 – 21.34) than at SA (19.67 – 25.08) and was significantly different in 1986 and 1991 (Table 2). The mean TD of earlywood cells of like years was not statistically different between the sites (χ^2 -test, $P > 0.05$) and varied from 20.62 μm to 21.96 μm for LA and from 20.25 μm to 22.44 μm for SA. The mean CWT of earlywood cells varied from 2.78 μm to 2.94 μm at LA and from 3.00 μm to 3.31 μm at SA, and differed significantly between sites in 1991 (Table 2). In the

latewood, LA also displayed lower number of cells per radial file (1.74 – 1.89) and thinner CWT (2.63 – 2.78 μm) than SA (respectively, 2.31 – 3.18; 3.04 – 3.38 μm). These differences were statistical different for al the years except for 1996. TD at SA (9.02 – 10.50 μm) was larger than TD at LA (8.46 – 9.08 μm), however the difference was only significant in 1979 (Table 2; significant effects of the factors considered in the GEEs model are showed in Appendix, Table A1).

Along the normalized tracheidogram TD gradually decreased from 26 to 7 μm , whereas CWT varied around 3 μm , increasing slightly toward the latewood cells (Fig. 4). In general, SA showed more intra-annual variability, especially for the years 1979 and 1986. Comparisons between site tracheidograms showed statistical differences among the years and positions for all parameters (χ^2 -test, $P < 0.001$); and difference between the sites was marginally significant for the ratio LD/CWT ($P = 0.064$; Table 3). Lower LD/CWT values were observed for SA due to smaller LD and thicker CWT. (For significant effects of the individual factors sex and size in the extended MLMs see Appendix, Table A2).

Relationship between cell size and water stress

During the selected years, trees at LA suffered less from water stress than those at SA. The water balance at LA is typically above 200 mm; only in the summers of 1986 and 1991 the water balance did drop below zero. In contrast, water availability at SA was always limited and never reached values greater than 80 mm. Drought stress in 1986 and 1991 was therefore more intense and persistent at SA than at LA (Fig. 5). A qualitative comparison of water balance patterns over the growing season and tracheid size deviation indicated some similarities between these two parameters. Despite the impossibility of synchronizing the two time-series – because of missing information about the timing of wood formation – it appeared that periods with a low water balance corresponded to smaller than average tracheids, and vice versa, a positive change in the water balance after rewatering given by strong precipitation corresponded to an increase in tracheid size (Fig. 5). This relationship

was especially manifest under dry conditions, i.e., for the dry SA site and for the drought summer years 1986 and 1991 of the mesic LA (Fig. 5).

DISCUSSION

The climate of inland areas of the western Mediterranean Basin is characterized by cold, wet winters and hot, dry summers. Cambial activity can therefore be subjected to two interruptions, one during the winter due to low temperatures and one during the summer triggered by water stress (Liphschitz and Lev-Yadun 1986, Cherubini et al. 2003). Therefore, depending on climate conditions of the year and on the autoecology of the species, differing seasonal patterns of secondary growth might occur (Camarero et al. 2010) which might also be able to affect the anatomical characteristics of the wooden cells formed during the growing season. Water stress is known to affect xylem growth directly and indirectly (Denne and Dodd 1981, Abe and Nakai 1999). In the early stage of water deficit, cell expansion is physically inhibited due to the loss of cell turgor (Abe et al. 2003). As water deficit continues, stress affects the whole plant physiology, reducing or preventing cell metabolism and indirectly limiting growth (Larson 1963, Arend and Fromm 2007).

The *J. thurifera* trees analysed exhibited a growth response characteristic of Mediterranean trees, namely reduced growth in years characterized by relatively dry and warm summer. This confirms that cambial activity is controlled mainly by water availability during the growing season in Mediterranean regions (Cherubini et al. 2003). However, growth responses differed between *J. thurifera* sites for the late winter and early spring conditions. Precipitation inhibited growth at the wetter and colder LA site, likely because snow and cold conditions delayed the start of the growing season. By contrast, late winter and early spring precipitation at the dry and relatively mild SA site supplied the water needed for growth initiation. Therefore, growth at LA was affected by two climatic limiting factors: temperature at the beginning of the growing season and drought during the summer, while growth at SA was regulated primarily by water availability.

As regard to the cell anatomical characteristics, the two sites exhibited similar cell anatomy, likely due to a strong genetic control of tracheid dimension (Willson et al. 2008). Although, we observed some clear differences between the sites and among the climatic years. Earlywood tracheids – which represented about 85 to 91% of the cells in the ring – showed lower LD/CWT values at the drier site (SA) than at the more mesic site (LA), whereas the intra-annual pattern of the wood cell anatomical characteristics was strongly affected by the within year climatic conditions. All analyzed cell anatomical characteristics significantly differed among the years and positions within the rings. In general, we observed that intra-annual unfavourable growing conditions, as water status reduction under drought conditions, fostered the production of smaller and thicker tracheids, and vice versa. In particular, years with severe early summer drought (1986 and 1991) were characterized by smaller LD and thicker CWT. In addition, thicker CWT in SA site also occurred for the latewood tracheids of the years with drier conditions in fall (1979, 1986 and 1991; Tables 1, 2). The observed results reinforce observations made for other species (Jenkins 1974, Nicholls and Waring 1977, Sheriff and Whitehead 1984), and are also consistent with the assumption that smaller and thicker cells are a safety adjustment against drought cavitation for conifers in limiting conditions (Cochard 1992, Willson and Jackson 2006, Eilmann et al. 2009).

The cell division in SA dry site might occur in pulses depending on rainfall events (Camarero et al. 2010) and be oriented in producing new larger size tracheids needed to guarantee for the water transport. These results support previous interpretations of the occurrence of intra-annual density fluctuations – through unusual cell anatomical pattern within the ring – observed in Mediterranean tree species which have been related to variability in the seasonal amount and distribution of precipitation (Rigling et al. 2001, Masiokas and Villalba 2004, Campelo et al. 2006, 2007, De Luis et al. 2007, Rossi et al. 2009).

Differences were also observed in regard to the number of tracheids, which was lower in LA (~18) than in SA (~25) both in the dry years 1986 and 1991. Large cell numbers, and subsequently wider ring-width, particularly in latewood, may result as a direct consequence of a longer period of cambial activity (Camarero et al. 2010) or as a mechanism to preserve enough conductive area overcoming the consequences of tracheid cavitation; albeit the latter strategy should be further explored in the future.

This set of results confirms that the course of the seasonal climatic conditions is reflected in the intra-annual pattern of cell anatomical features. Although this study cannot directly link water balance to cell size – due to the lack of on-line timing of xylogenesis and in situ water stress measurements – there are however strong indications that these deviations in cell anatomical characteristics from the “regular pattern” can be an indicator of the tree water status over the growing season. In particular for the dry site (SA), where growth was strongly limited by water stress, we observed very similar intra-annual patterns of water balance and cell size (Figure 5). Similar behaviour is observable also for the more mesic site (LA); however this relationship appears to be weaker and limited only to the years characterized by strong summer stress such as 1986 and 1991. Therefore we suggest that, if drought is the main limiting factor over the whole growing season, deviation from average tracheidogram might be a function of water availability, as it has been previously described for other physiological activities in arid environments (Huxman et al. 2004).

In conclusion, the results obtained from this explorative study indicated that, differences in cell anatomical characteristics between the dry and mesic sites of *Juniperus thurifera* reflected site-specific adjustments; but also under dry conditions there was a strong influence of the seasonal water availability on the intra-annual anatomical cell pattern. Specifically, those periods characterized by dry conditions seemed to promote the formation of smaller and thicker cells and vice versa. Thus, through a correct site selection, there is

- 298 potential for using series of intra-annual wood anatomical features, particularly earlywood
- 299 series, to retrospectively relate to the seasonal climatic pattern.

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FIGURES

Fig. 1. a) Distribution area of *Juniperus thurifera* (grey regions) in the Iberian Peninsula; locations of Peña Lampa (LA) and Santa Engracia (SA) sites (solid circles); and derived climate diagrams, b), precipitation (solid line), potential evapotranspiration (PET, dashed line) and water balance (WB, dotted line). Climate parameters were calculated over the period 1961-2002 obtained from the CRU dataset of the corresponding 0.5° grid

Fig. 2. Procedure for the cell anatomical measurements. a) Image of an analyzed cross-section with indication of the radial files (arrows lines) selected for the measurements. b) Intensity values of a single line along a radial file (line profile). c) Schema indicating how the measured anatomical variables have been calculated. LD = radial lumen diameter, DWT = double cell wall thickness, CWT = cell wall thickness, TD = tracheid diameter

Fig. 3. a) Site chronologies and b) growth responses to climate. LA = Peña Lampa; SA = Santa Engracia. Black (LA) and grey (SA) lines indicate mean radial width of all individuals sampled in the stands. Arrows indicate the annual rings selected for cell anatomical analyses. Included data correspond to selected sampled trees for the period 1951–2006. Climate-growth relationships are calculated with monthly average temperature and sum precipitation over the period 1961–2002 from the climatic stations of Zaragoza Aula Dei (SA) and Velilla del Rio Carrion (LA)

Fig. 4. Site tracheidograms for each anatomical variable and selected climatic year. TD = radial tracheid diameter, LD = radial lumen diameter, CWT = radial cell wall thickness, LD/CWT = ratio lumen diameter to cell wall thickness. LA = Peña Lampa (n=8), SA = Santa Engracia (n=6). The number of cell per rings has been normalized to 20 in earlywood (EW) + 3 in latewood (LW). Symbols indicate the mean and vertical bars the standard deviation. Lines in TD tracheidogramm indicate the average tracheidogramm (fourth order polynomial fitting)

Fig. 5. Relationships between deviation from average site tracheidogram of tracheid diameter (black dots) and water balance (line) for each selected climatic year. LA = Peña Lampa, SA = Santa Engracia. Lines indicate decade of water balance starting from March to the end of November for every year and site. LA = Peña Lampa (climate station of Velilla del Rio

Carrion); SA = Santa Engracia (climate station of Zaragoza Aula Dei). Xilogenesis timing in the figure is in accordance to a previous study with this species in the Iberian Peninsula (Camarero et al. 2010)

Figure 1

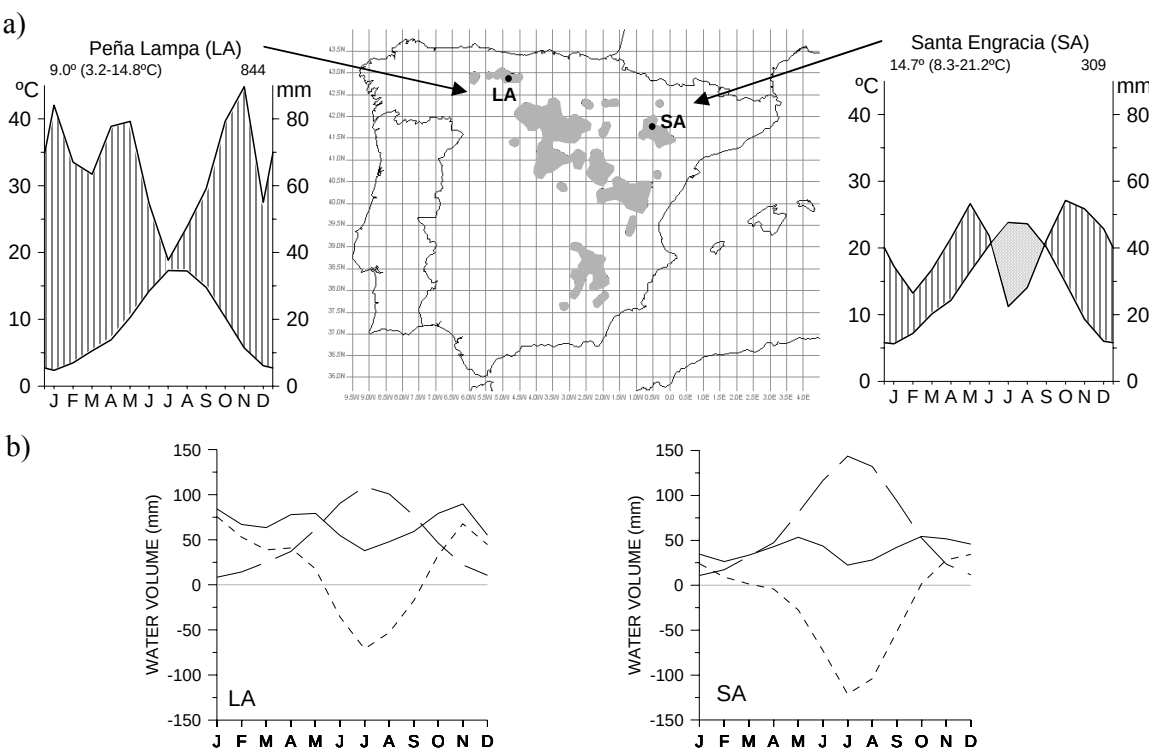


Figure 2.

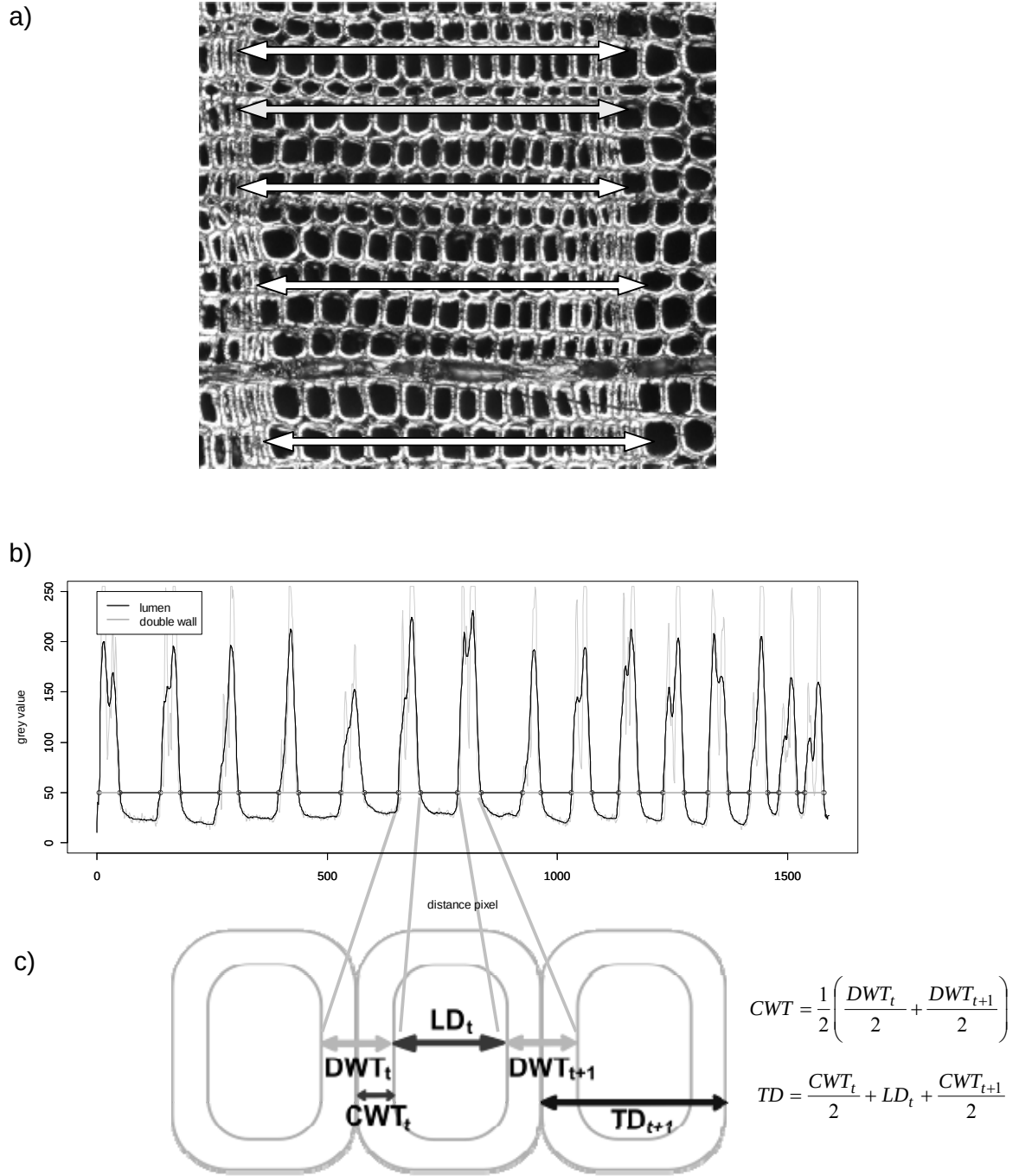


Figure 3.

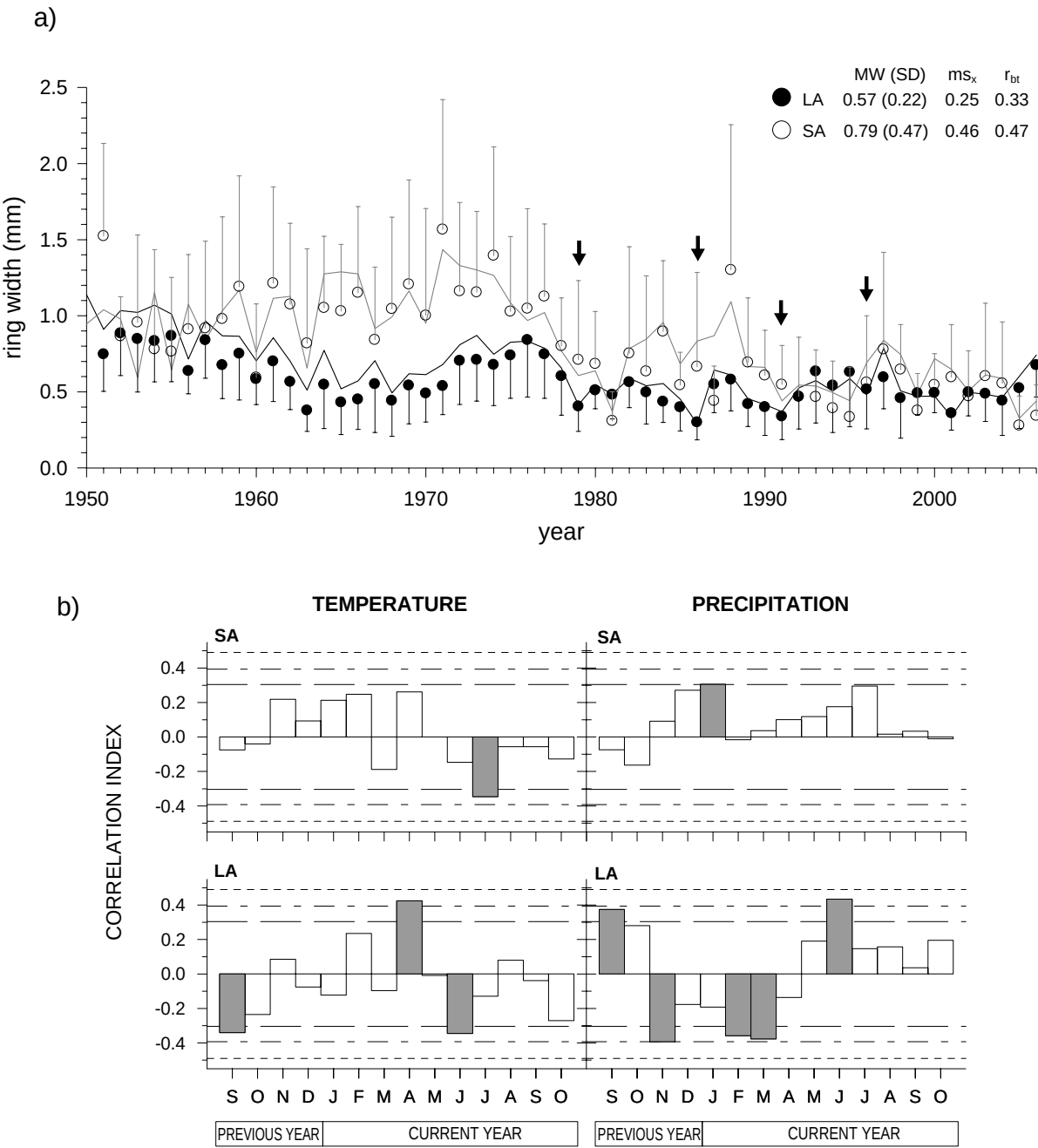


Figure 4.

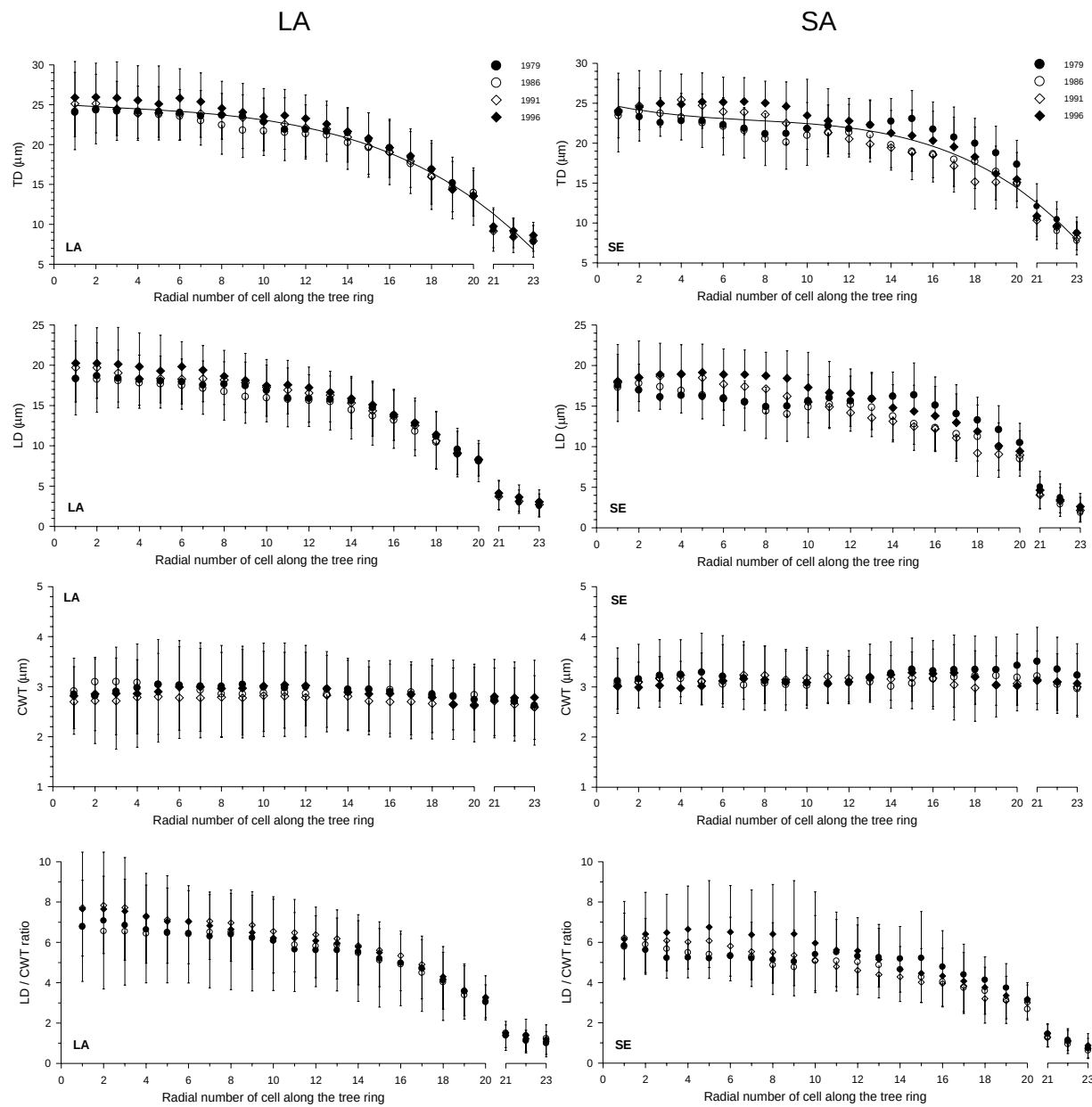
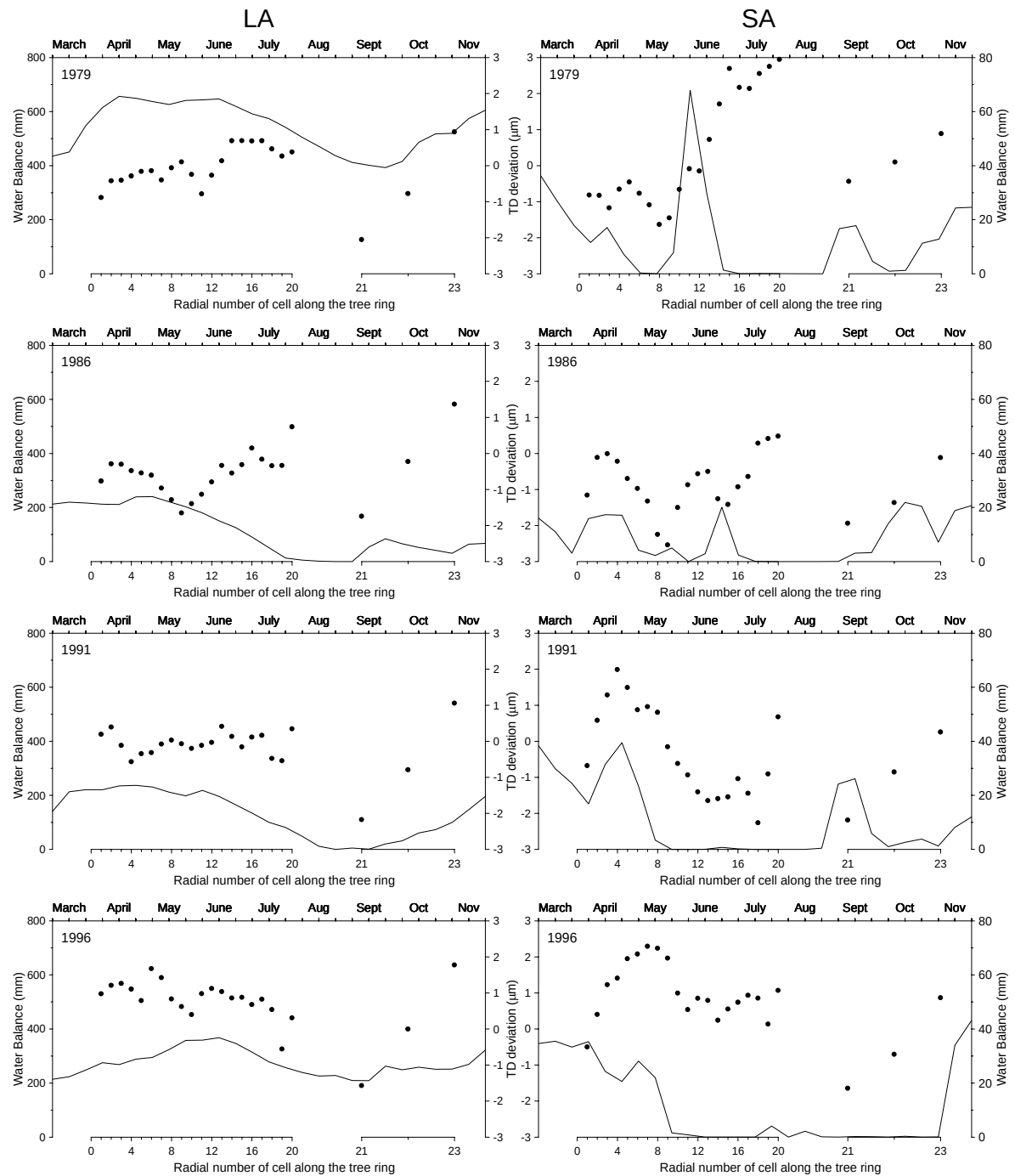


Figure 5.



TABLES

Table 1. Monthly, seasonal and yearly deviance of precipitation for the 4 selected climatic year compared to the long-term average over the period 1951–2000.

	J	F	M	A	M	J	J	A	S	O	N	D	Winter	Spring	Summer	Fall	Year
LA																	
1979	98	188	108	-9	-35	-10	-8	-100	-33	72	-21	-10	130	-20	-41	10	36
1986	-13	72	-74	-43	-67	-88	-91	1	101	-88	-60	-63	-3	-65	28	-69	-35
1991	-7	-31	92	-37	-53	-64	5	-96	33	-23	-2	-98	13	-50	-3	-41	-20
1996	179	-15	-4	-26	23	5	-25	132	54	-71	-19	-1	64	3	51	-28	19
SA																	
1979	191	28	-74	-12	113	-45	-30	-60	72	42	-33	-58	46	27	12	-12	17
1986	-57	13	-60	35	0	44	25	-79	24	91	-20	-46	-36	24	-3	14	4
1991	-86	138	-30	35	-82	-52	1	-28	152	-28	-12	-64	4	-37	68	-32	-6
1996	278	26	35	-17	14	-60	78	30	-78	-87	68	177	113	-18	-11	40	26

Note: Values are in [%]. SA = Santa Engracia (climate station of Zaragoza Aula Dei); LA = Peña Lampa (climate station of Velilla del Rio Carrion). Winter = JFM; Spring = AMJ, Summer = JAS, Fall = OND

Table 2. Least-square mean (Mean) and standard error (SE) of wood anatomy features of earlywood, latewood and total ring cells for each studied year.

		1979			1986			1991			1996		
Wood features	Site	Mean	SE	χ^2	Mean	SE	χ^2	Mean	SE	χ^2	Mean	SE	χ^2
Earlywood													
# Cells	LA	21.34 ^{ac}	1.15	1.63	13.81 ^{bc}	1.17	8.81 ^{**}	12.86 ^c	1.24	4.42 [*]	17.77 ^{ab}	1.22	0.01
	SA	25.08 ^a	1.17		20.50 ^b	1.09		22.73 ^{ab}	1.29		19.67 ^{ab}	1.34	
RW	LA	413.43 ^{ac}	1.20	1.41	270.40 ^{bc}	1.22	6.39 [*]	257.45 ^c	1.20	3.33	340.96 ^{ab}	1.19	0.30
	SA	470.11 ^a	1.23		396.28 ^{ab}	1.10		414.76 ^{ab}	1.29		344.67 ^b	1.36	
LD	LA	15.63 ^a	0.60	0.78	14.99 ^a	0.71	1.04	16.16 ^b	0.43	4.39 [*]	16.14 ^{ab}	0.54	0.05
	SA	14.99 ^a	0.42		14.18 ^a	0.47		14.48 ^a	0.35		16.03 ^b	0.76	
CWT	LA	2.94	0.11	3.38	2.80	0.20	1.37	2.78	0.19	5.95 [*]	2.91	0.17	1.51
	SA	3.31 ^a	0.13		3.00 ^b	0.06		3.08 ^{ab}	0.05		3.23 ^b	0.12	
TD	LA	21.54	0.59	0.03	20.62	0.67	0.20	21.68	0.54	0.40	21.96	0.61	0.05
	SA	21.61 ^a	0.37		20.25 ^b	0.39		20.69 ^b	0.31		22.44 ^a	0.83	
Latewood													
# Cells	LA	1.80	1.13	9.58 ^{**}	1.89	1.17	9.53 ^{**}	1.74	1.15	7.59 ^{**}	1.84	1.16	0.98
	SA	2.55 ^a	1.10		3.18 ^a	1.06		2.79 ^a	1.08		2.31 ^b	1.13	
RW	LA	13.42	1.14	15.42 ^{***}	14.08	1.23	7.61 ^{**}	12.46	1.16	8.54 ^{**}	14.29	1.16	2.15
	SA	23.12 ^a	1.14		24.46 ^a	1.07		23.18 ^{ab}	1.12		19.82 ^b	1.14	
LD	LA	3.27 ^a	0.16	5.80 [*]	3.45 ^{ab}	0.22	3.48	3.19 ^{ab}	0.18	0.00	3.52 ^b	0.21	0.03
	SA	3.77 ^a	0.17		2.92 ^b	0.14		3.19 ^{bc}	0.16		3.58 ^{ac}	0.28	
CWT	LA	2.72 ^{ab}	0.14	18.54 ^{***}	2.69 ^{ab}	0.15	6.39 [*]	2.63 ^a	0.13	7.22 ^{**}	2.78 ^b	0.14	3.27
	SA	3.38 ^a	0.10		3.04 ^b	0.03		3.08 ^b	0.10		3.09 ^b	0.11	
TD	LA	8.69	0.39	14.72 ^{***}	8.84	0.50	0.23	8.46	0.36	3.52	9.08	0.42	1.37
	SA	10.50 ^a	0.36		9.02 ^b	0.15		9.35 ^b	0.30		9.75 ^{ab}	0.47	
Total ring													
# Cells	LA	22.65 ^{ac}	1.14	2.12	15.66 ^{bc}	1.16	9.63 ^{**}	14.97 ^c	1.21	5.07 [*]	19.91 ^{ac}	1.20	0.00
	SA	27.34	1.15		23.93	1.08		25.93	1.25		22.16	1.31	
RW	LA	426.48 ^{ac}	1.18	1.78	288.40 ^{bc}	1.21	6.69 [*]	275.49 ^c	1.18	4.15	360.66 ^{ab}	1.19	0.22
	SA	500.38	1.20		428.06	1.09		450.51	1.27		367.71	1.35	
LD	LA	14.60	0.70	0.76	13.78	0.84	0.68	14.50	0.44	2.36	14.80	0.50	0.24
	SA	13.76 ^a	0.60		12.78 ^b	0.42		12.98 ^b	0.56		14.40 ^a	0.91	
CWT	LA	2.92	0.11	4.67 [*]	2.78	0.19	1.94	2.76	0.17	7.35 ^{**}	2.90	0.16	1.46
	SA	3.32 ^a	0.13		3.00 ^b	0.05		3.09 ^{ab}	0.05		3.21 ^b	0.11	
TD	LA	20.42	0.67	0.02	19.32	0.74	0.04	20.04	0.53	0.10	20.66	0.58	0.01
	SA	20.41 ^a	0.54		18.76 ^b	0.34		19.17 ^b	0.52		20.84 ^a	1.07	
% LW	LA	9.69 ^{ac}	1.16	0.32	12.49 ^b	1.16	0.00	12.30 ^{ab}	1.12	0.28	9.25 ^c	1.09	4.94 [*]
	SA	11.92 ^a	1.24		13.60 ^{ab}	1.06		12.93 ^b	1.21		13.69 ^{ab}	1.17	

Note: Data refers to 8 trees (13 radii) in Peña Lampa (LA) and 6 trees (10 radii) in Santa Engracia (SA). Five radial files of cells are measured for each ring (for a total of 460 files). Least-square mean significant differences between sites are indicated with asterisks (χ^2 test; * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$) and among years are indicated with different letters (χ^2 test; $P < 0.05$). Values for ring width (RW) lumen diameter (LD), cell wall thickness (CWT) and tracheid diameter (TD) are expressed in μm .

Table 3. Type III test conducted on the multilevel mixed model to test the effect of the fixed factors “Year”, “Site” and “Cell position” within the tracheidogram on the wood anatomical features.

Factor	df num	df den	LD		CWT		TD		LD/CWT	
			<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
EW										
site	1	13	1.28	0.278	2.84	0.116	0.02	0.883	4.10	0.064
year	3	39	123.22	< 0.001	23.41	< 0.001	104.89	< 0.001	54.96	< 0.001
position	19	247	466.59	< 0.001	1.8	0.023	400.95	< 0.001	223.02	< 0.001
site × year	3	39	9.02	< 0.001	14.40	< 0.001	5.31	0.004	25.74	< 0.001
site × position	19	247	8.41	< 0.001	3.47	< 0.001	10.52	< 0.001	4.72	< 0.001
year × position	57	798	5.39	< 0.001	1.37	0.040	5.3	< 0.001	2.33	< 0.001
LW										
site	1	13	0.01	0.941	6.33	0.026	3.83	0.072	8.25	0.013
year	3	39	4.83	0.006	6.09	0.002	6.52	0.001	3.93	0.015
position	2	26	133.75	< 0.001	7.48	0.003	93.91	< 0.001	72.3	< 0.001
site × year	3	39	8.49	< 0.001	6.89	0.001	10.53	< 0.001	5.9	0.002
site × position	2	26	16.11	< 0.001	0.55	0.586	10.5	< 0.001	8.31	0.002
year × position	6	84	0.64	0.694	0.55	0.769	0.67	0.674	0.23	0.964

Note: LD = Lumen diameter, CWT = cell wall thickness, TD = tracheid diameter and LD/CWT ratio lumen diameter to cell wall thickness. Data represent numerator (df num) and denominator degrees (df den) of freedom, the *F*-statistic (*F*) and *P*-value of significance (*P*). Significant effects are in bold ($P < 0.05$). $n = 9,600$ for earlywood (EW), $n = 1,440$ for latewood (LW) and number of radial files = 460.