MISSING AND DARK RINGS ASSOCIATED WITH DROUGHT IN *PINUS HALEPENSIS*

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ABSTRACT

The responses of the vascular cambium and tracheid differentiation to extreme drought in Aleppo pine (*Pinus halepensis* Mill.) were investigated. The research focused on the drought year of 2005, in the primary study area at Maigmo (MAI) in southeastern Spain, with comparisons in Jarafuel (JAL) and Guardamar (GUA). The climate in this region is typically warm and dry with hot summers. Wood formation throughout the 2005 growing season was studied in transverse microtome sections and integrated with a retrospective dendrochronological analysis of crossdated increment cores collected in 2009. For most anatomical sections collected throughout the growing season at MAI, the vascular cambium appeared to be dormant as indicated by the low number of cells per radial file. Occasionally, immature xylem derivatives were observed during the growing season but without production of an annual ring. In increment cores collected at MAI, the 2005 position in the annual ring series contained either a narrow ring of both earlywood and latewood (47 % of samples), a narrow ring of apparent latewood with no earlywood (13 %), or a missing ring (50 %). We introduce the term "dark ring" to refer to those annual rings of apparent latewood with no earlywood. For trees at JAL, the 2005 ring had below-average width and contained both earlywood and latewood. At GUA, the trees produced the widest 2005 ring of all three sites and mainly contained an intra-annual density fluctuation (IADF). The IADF was formed after cambial reactivation in the autumn. Although dark rings, IADFs, and especially missing rings complicate dendrochronological analysis, these anatomical features may provide an additional proxy record from which to infer climate variability and change in the past.

Key words: Aleppo pine, wood formation, missing ring, dark ring, false ring, intra-annual density fluctuation, Spain.

INTRODUCTION

Dendrochronology is the science of assigning calendar year dates to annual tree rings (Kaennel & Schweingruber 1995). Dendrochronology is particularly useful to investigate environmental influences on the growth of trees from temperate, arid, alpine, and arctic climatic zones. In many conifer species, annual ring boundaries are seen as the discontinuity between the relatively thin-walled and light-coloured earlywood and the thick-walled and dark-coloured latewood of the previous year (*e.g.* Schweingruber 1983). Under extreme stress, cell division by the vascular cambium may not occur, resulting in the lack of formation of an annual ring. This absence in the series of tree rings is commonly referred to as a missing ring. Missing rings may more appropriately be referred to as locally absent rings, as rarely if ever is a ring missing from all radii of a given cross section or from all possible crosssections along a woody stem (Novak *et al.* 2011).

Missing rings may result from different stresses such as drought, cold, or nutrient deficiency (*e.g.* Liang *et al.* 2006; Novak *et al.* 2011; Liang *et al.* 2014), pollution and dieback (*e.g.* Sander *et al.* 1995; Bigler *et al.* 2004; Malik *et al.* 2012; Bräuning *et al.* 2016), insect outbreak (*e.g.* Sangüesa-Barreda *et al.* 2014; Robson *et al.* 2015), or fungal infection (*e.g.* Cherubini *et al.* 2002). They may be especially frequent in species growing at their distribution limits, where the growth rates are generally low (*e.g.* Wilmking *et al.* 2012; Dulamsuren *et al.* 2013; Qin *et al.* 2013). Although the frequency of occurrence varies, missing rings appear to be a general strategic response to extreme conditions for a wide variety of tree species, growth forms and environments (*e.g.* Novak *et al.* 2011; Wilmking *et al.* 2012; Liang *et al.* 2014). The position of a missing ring along a series of tree rings can be identified through precise and accurate crossdating of multiple tree-ring series. Although missing rings cannot provide data on the usual characteristics of ring width, density, etc., missing rings do indicate reduced cambial activity attributable to environmental factors and ecophysiological processes.

In recent years, several studies have investigated environmental signals recorded in wood anatomy and tree-ring characteristics (*e.g.* Čufar *et al.* 2008; Fonti *et al.* 2010; De Luis *et al.* 2011a, b; Novak *et al.* 2013a, b; Beeckman 2016; Giagli *et al.* 2016; Gričar *et al.* 2016). Repeated sampling of vascular cambium and differentiating secondary xylem throughout the growing season has identified stages of formation of more or less normal rings (*e.g.* Deslauriers *et al.* 2008; Rossi *et al.* 2013; Vieira *et al.* 2014) as well as those containing various anomalies, such as intra-annual density fluctuations (*e.g.* Vieira *et al.* 2010; De Luis *et al.* 2011a, b; Novak *et al.* 2013a, b; Campelo *et al.* 2015). Numerous studies have tried to link seasonal rhythm of cambial activity and wood formation with environmental factors (*e.g.* Begum *et al.* 2013; Li *et al.* 2013). However, details are lacking for situations in which cambial production of xylem was extremely low or absent during the entire growth period.

The annual rhythm of cambial activity in Mediterranean ecosystems is much more complex than for trees in more mesic temperate environments (Cherubini *et al.* 2003). Under some conditions, Mediterranean trees undergo continuous cambial divisions throughout the year. Alternatively cambial division may be interrupted during winter,

mainly due to low temperatures and short photoperiod as well as during summer, due
to high temperatures and lack of precipitation (*e.g.* Lev-Yadun 2000; Nicault *et al.*
2001; De Luis *et al.* 2007*,* 2011a) or the growth cycle may even begin in autumn and
continue through the winter as for some woody shrubs (Cherubini *et al.* 2003). The
high frequency of missing rings observed in Mediterranean trees (*e.g.* Raventós *et al.*
2001; Novak *et al.* 2011) is likely due to the increased frequency of extremely low
amounts of precipitation (*e.g.* Vicente-Serrano *et al.* 2015).

Aleppo pine (*Pinus halepensis* Mill.) is an important and widespread tree species
in the Mediterranean area, growing under widely diverse climatic and edaphic con-
ditions (Barbéro *et al.* 1998; Richardson & Rundel 1998). Aleppo pine is tolerant of
high temperatures and drought and does not tolerate shade, excessive humidity, or
frost and snow (Girard *et al.* 2012). Due to its growth plasticity and adaptability to dif-
ferent site and climatic conditions, Aleppo pine is an important species to monitor for
the effects of climatic change on trees across the Mediterranean (De Luis *et al.* 2013;
Olivar *et al.* 2015).

The coastal belt of southeastern Spain including forest sites of Aleppo pine expe-
rienced an extreme drought in 2005. These sites are characterized by a low annual
sum of precipitation (300–400 mm), high mean annual temperatures (14–17 °C), and
pronounced summer droughts. We expected that the particularly severe 2005 drought
could result in reduced cambial growth and missing rings in Aleppo pine. We also
expected that missing rings and dendrochronological characteristics would be affected
by the distance from the coast, altitude, temperature, and precipitation.

The objective of this research is to test those expectations and to investigate the
intra-annual dynamics of cambial productivity and xylem formation in Aleppo pine
at Maigmo and comparable sites in southeastern Spain during 2005, a year character-
ized by extreme drought. The investigation combines microanatomical analysis of
samples collected during the growing season with a retrospective dendrochronological
analysis.

MATERIALS AND METHODS

Study sites and climatic conditions

Cambial productivity and xylem formation of Aleppo pine (*Pinus halepensis* Mill.)
was investigated in southeastern Spain. The primary study area was a naturally re-
generated and uneven-aged stand in Maigmo (MAI) in Alicante Province (Fig. 1 & 2;
Table 1). The typical climate at MAI is moderately warm and dry with most of the an-
nual rainfall occurring in spring and in autumn, separated by a dry summer (De Luis
et al. 2011a; Novak *et al.* 2013b). For comparison, observations were made of Aleppo
pine at Jarafuel (JAL), located more inland than MAI and from previously reported
observations from trees at Guardamar del Segura (GUA), a sand dune ecosystem lo-
cated on the coast (De Luis *et al.* 2011a) (Fig. 1 & 2; Table 1).

A review of monthly mean temperature and total precipitation for 2005 at MAI
indicates a somewhat warmer and much drier year until October than the 1901–2013
average (Fig. 2a). Although climatic conditions at JAL and GUA for 2005 were gen-
erally similar, JAL experienced a greater total precipitation for September and close
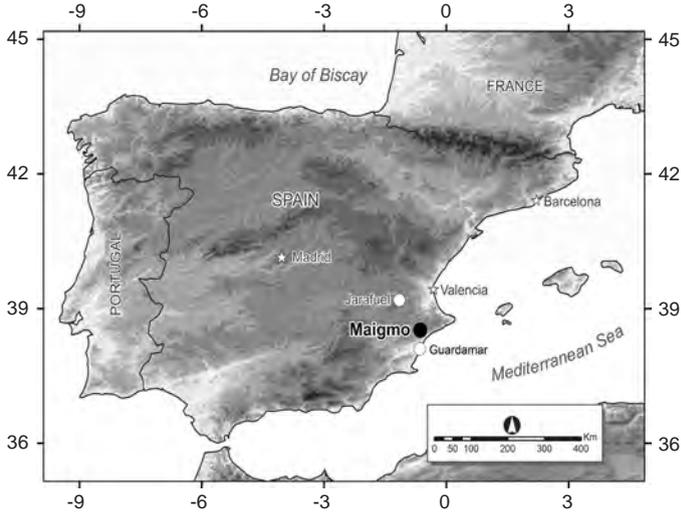
Figure 1. Study areas Maigmo, Guardamar, and Jarafuel in southeastern Spain.

to the long-term average for October in 2005 (Fig 2b, c). At GUA, the long-term average temperature is higher and the total precipitation is lower throughout the year. At GUA, the total precipitation for August and September met or slightly exceeded the long-term mean followed by a dry October and wet November 2005.

Cambial productivity and xylem formation

We selected 6 dominant or co-dominant Aleppo pine trees for tissue sampling at each location (MAI, JAL, GUA). On all sites the trees were about 80 years of age and 40 cm in diameter at breast height with no obvious signs of damage or disease. Tissue sample blocks were collected at biweekly intervals from February through December, 2005.

Intact blocks of phloem, cambium, and outer xylem ($25 \times 10 \times 10$ mm) were taken with a chisel and cutter knife at breast height from each sample tree. In order to avoid the effect of wounding the distance between neighbouring samples was at least 10 cm, so that the new sample would not contain traumatic tissue or wound-wood, which can be formed as a response of the cambium to mechanical damage (Gričar *et al.* 2007).

Table 1. Geographical coordinates and mean annual temperature and total precipitation for the study areas in southeastern Spain (1901–2013, calculated from Harris *et al.* 2014).

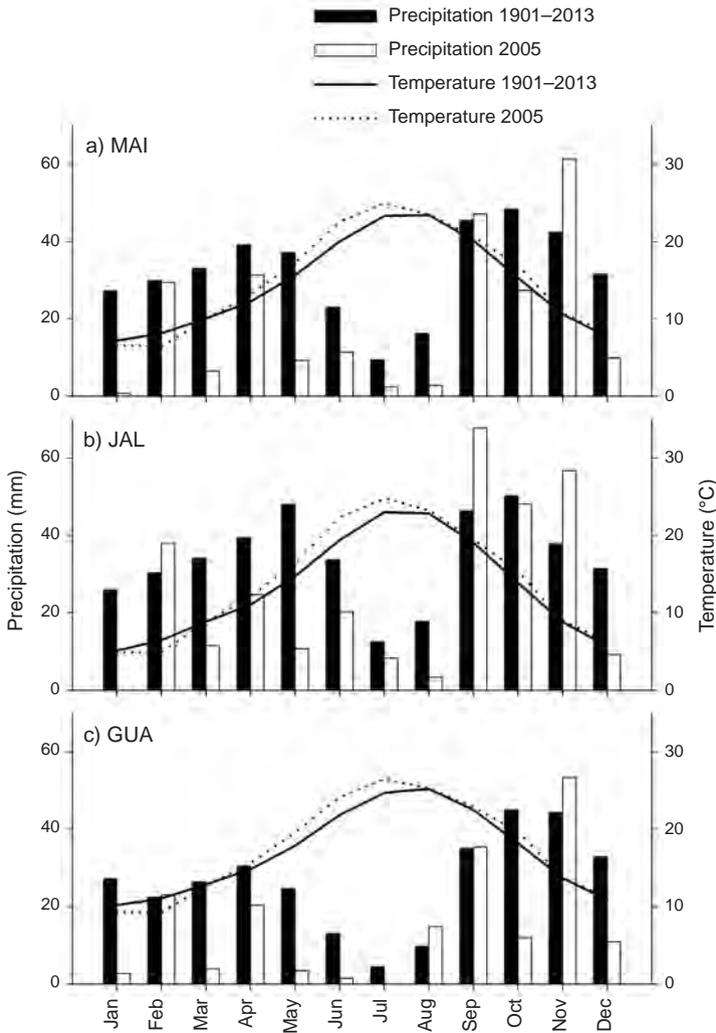| Location	Latitude	Longitude	Elevation (m)	Temperature (°C)	Precipitation (mm)
Maigmo (MAI)	38° 03' N	0° 38' W	845	14.5	383
Jarafuel (JAL)	39° 08' N	1° 04' W	571	14.0	407
Guardamar (GUA)	38° 06' N	0° 40' W	15	17.0	316

Figure 2. Climatic diagrams of a) Maigmo (MAI), b) Jarafuel (JAL), and c) Guardamar (GUA) obtained from climatic time series data from the Climatic Research Unit (CRU) of the University of East Anglia for 1901–2013 and for 2005.

Because of this sampling distance along the stem, blocks were not systematically collected with respect to compass direction.

Blocks were immediately fixed in FAA (formalin-ethanol-acetic acid) and subsequently dehydrated in a graded series of ethanol (30 %, 50 %, and 70 %). The sample blocks were trimmed (2 × 2 × 3 mm) and embedded in paraffin using a Leica TP 1020-1 tissue processor configured for dehydration in a graded series of ethanol (70 %, 90 %, 95 %, and 100 %) and Bio-clear (D-limonene) followed by paraffin infiltration (Rossi *et al.* 2006). Cross sections were cut to a thickness of 12 μm with a Leica RM 2245

rotary microtome fitted with disposable Feather N35H blades. To improve section adhesion, glass slides were pre-treated with albumin. Sections were dried at 70 °C for 30 min and cleaned of residual paraffin by immersing the slides in Bio-clear and ethanol. Sections were double-stained, first with safranin (0.5 % in 95 % ethanol) and then with astrablue (0.5 % in 95 % ethanol), and mounted in Euparal resin. Tissues and cells were examined and recorded with a Nikon Eclipse 800 light microscope (bright field and polarized light) fitted with a DS-Fi1 digital camera and the NIS-Elements BR 3 image analysis system.

Cambial cells and tracheids in the wood were identified using visual criteria of proportional dimension and wall thickness (De Luis *et al.* 2007, 2011a). Numbers of cells were counted along three radial files for cambial cells (CC), expanding post-cambial xylem derivatives (PC), immature xylem derivatives with thickening secondary walls (SW), and mature tracheids (MT). Active production of xylem cells by the cambium was indicated by an increase in the number of CC and the presence of PC.

Retrospective dendrochronology

As no missing rings were observed at JAL and GUA, the tree ring of the year 2005 was inspected in samples of the 6 trees per site used to study wood formation. At MAI, where missing rings were identified, duplicate increment cores were taken at breast height from an additional 20 trees in December 2009. The cores were air-dried, mounted in grooved wooden blocks, and surfaced with a graded series of sandpaper. Surfaced cores were photographed and digital images were examined in Serif Photoplus SE. Tree-ring series were visually crossdated by comparison of signature rings and calendar dates assigned to the year of formation of each ring. The quality of the crossdating was checked by COFECHA software (Holmes 1994). Tree-ring widths were measured to the nearest 0.01 mm using a LINTAB measuring table and TSAP measurement software (RINNTECH, Heidelberg, Germany).

RESULTS

Intra-annual and site comparisons of cambial productivity

The developmental stages of wood formation were assessed for Aleppo pine on three sites in 2005 (Fig. 3, 4, 5, 6). Presented here are new data for trees sampled at the primary study site at MAI and the comparison site at JAL with new presentations of previously published data from GUA (De Luis *et al.* 2011a). Throughout 2005, the cambium of Aleppo pine at MAI contained on average of about 5.0–7.0 cells per radial file with no apparent seasonal trend in number (Fig. 3a). The average number of CC varied from about 5.5–8.5 at GUA and 3.5–7.0 at JAL with a period of seasonal depression in numbers from late May to early October. The zone of PC growth contained a sustained average of 1–2 cells at MAI through late July (Fig. 3b). The PC zone contained more cells per radial file at GUA and JAL with spring peaks of an average 5.5 and 7.0 cells, respectively.

Individual SW cells at MAI were occasionally observed from the end of May till the end of December with an average number of 0.5 in this period. The number of SW
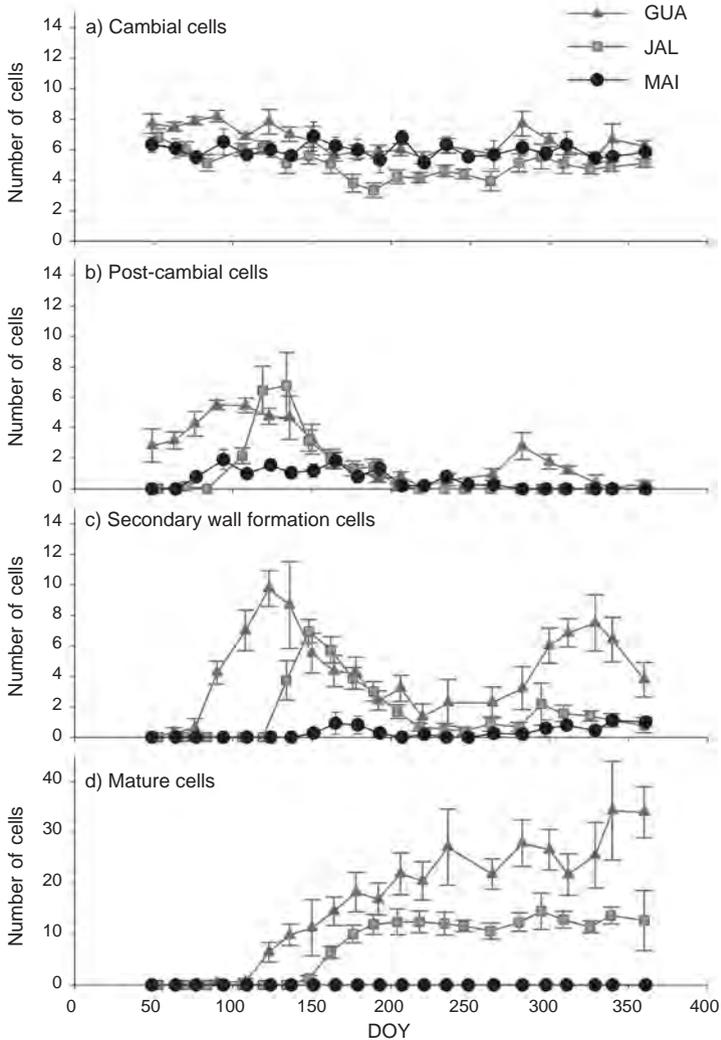
Figure 3. Wood formation dynamics at Maigmo (MAI), Guardamar (GUA), and Jarafuel (JAL) in 2005: a) average numbers and standard deviations of cambial cells (CC) per radial file, b) xylem cells in post-cambial growth (PC), c) tracheids undergoing secondary wall thickening and lignification (SW), and d) mature tracheids (MT).

at GUA and JAL peaked in the spring with averages of 10.0 and 7.0 cells, respectively (Fig. 3c). The SW zone at GUA contained an additional autumn peak of an average of about 7.0 cells. No mature tracheids (MT) were observed in tissue sections from MAI during 2005 or in some cases we could not establish if they were formed in 2004 or 2005. First mature tracheids were present at GUA beginning in late April and at JAL beginning in early June of 2005 (Fig. 3d).
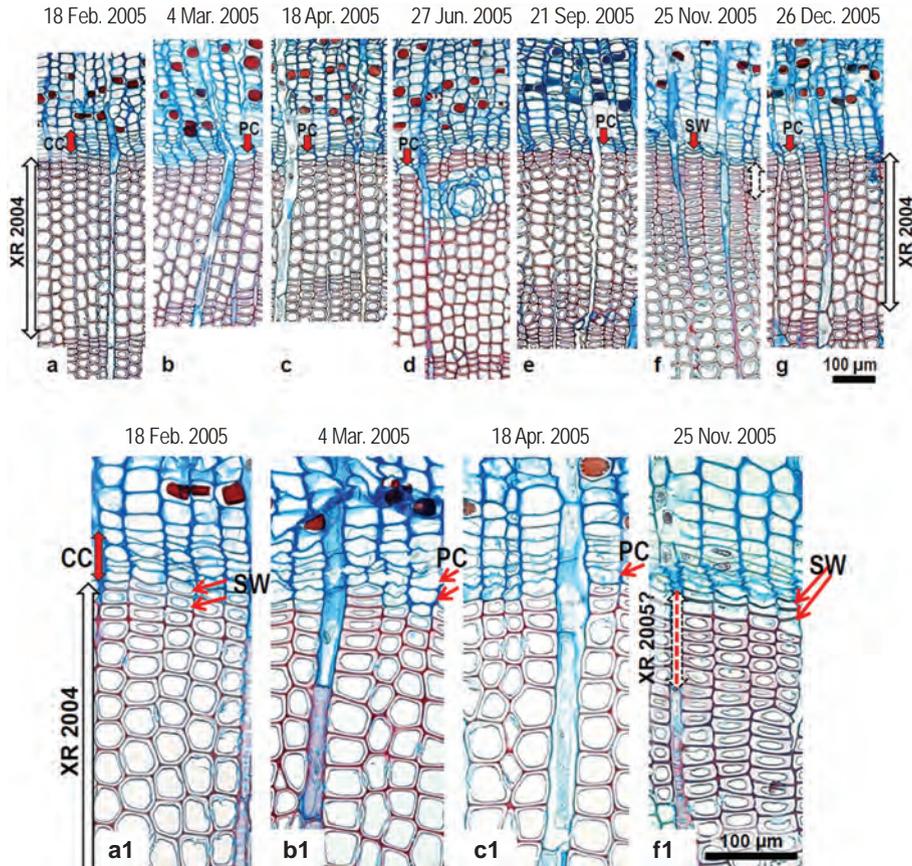
Figure 4. Time-course of cambial structure in a representative *Pinus halepensis* at Maigmo (MAI) (bark at top of section): (a–g) Cambial cells (CC), post-cambial derivatives (PC), and cells undergoing thickening of the secondary wall (SW) are marked. Mature xylem under CC was formed as part of the 2004 growth ring (XR 2004). (a1, b1, c1, f1) Detailed views of (a, b, c, and f). (a1) cells from 2004 completing secondary wall thickening in 2005 (SW); (b1, c1) PC cells (arrows); (f1) 2005 SW and latewood potentially formed in 2005 (XR 2005?) in the absence of 2005 earlywood.

The time-course of anatomical features for Aleppo pine at MAI in February 2005 shows the last-formed tracheids in the 2004 growth ring in SW phase (Fig. 4a: a1). Individual PC cells can be seen on samples taken from March until December (Fig. 4b–g, b1, c1). The completion of secondary wall thickening and lignification (SW) could be observed only on the samples taken at the end of November (Fig. 4f: f1); here we could also recognize latewood-like cells potentially formed in 2005 (XR 2005?), whereas 2005 earlywood seemed to be absent.

Samples collected from JAL in 2005 showed an onset of cambial cell division in early spring as indicated by PC formed to the outside of the 2004 latewood (Fig. 5a).
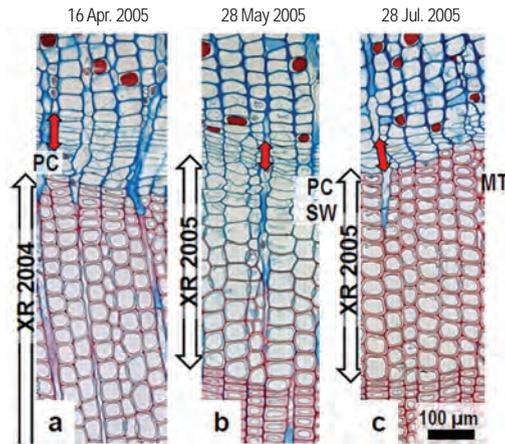
Figure 5. Wood formation in *Pinus halapensis* at Jarafuel (JAR): a) Cambium (red arrows) and post-cambial cells (PC) produced in 2005 to the outside of latewood of the 2004 growth ring (XR 2004); b) PC and cells with secondary wall thickening (SW) in the developing 2005 growth ring (XR 2005); c) Mature tracheids (MT) in the developing XR 2005.
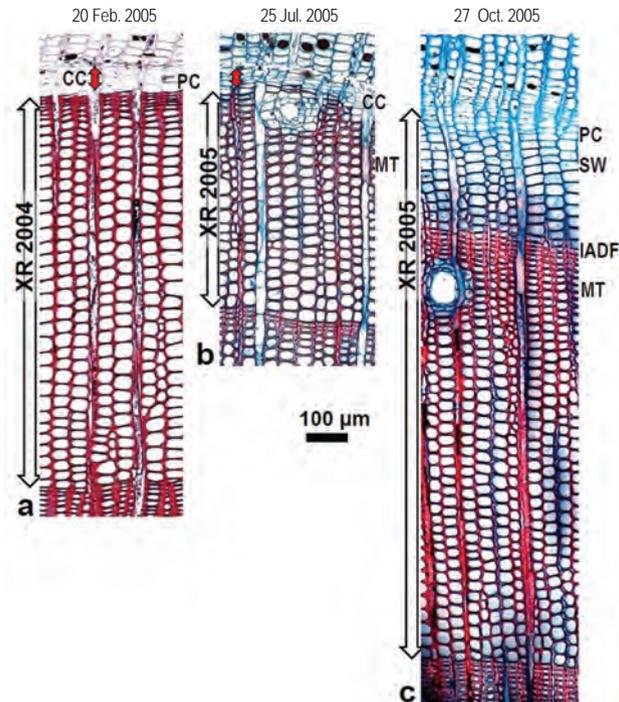

Figure 6. Wood formation in *Pinus halepensis* at Guardamar (GUA): a) The cambial cells (CC, red arrows) and post-cambial cells (PC) formed in 2005 to the outside of the 2004 growth ring (XR2004); b) Mature tracheids (MT) with no PC in the 2005 growth ring (XR2005); c) PC and cells with secondary wall thickening (SW) produced to the outside of the latewood MT and the intra-annual density fluctuation (IADF) (modified from De Luis *et al.* 2011a).

By late spring, SW cells had occurred along with the PC in a developing 2005 growth ring (Fig. 5b). By mid-summer, sections contained MT which mainly had characteristics of earlywood (Fig. 5c).

At GUA in 2005, the first PC cells were present in February (Fig. 4b, 6a) and mature latewood was present at mid-summer (Fig. 6b). Production of xylem cells essentially ceased from mid-June to early September as indicated by the absence of PC (Fig. 4b). By mid-September, PC cells were again present at GUA. The first cells formed during this second growth period in 2005 matured and appeared primarily as earlywood (Fig. 6c).

Retrospective evaluation of 2005 missing ring at MAI

Of the 38 crossdated tree-ring series collected from MAI in 2009, 19 (50%) were missing the 2005 growth ring (Fig. 7a). Rings containing normal earlywood and latewood for 2005 occurred in 14 of the series (37%) and averaged 0.36 mm in width. Rings apparently containing only latewood for 2005 occurred in 5 of the series (13%) and averaged 0.16 mm in width. These latewood-only rings appeared dark in reflected light and are here termed "dark rings". The boundaries between dark rings and adjacent rings formed the previous and following years were less distinct than normal ring boundaries (Fig. 7b). The duplicate cores taken from a single tree frequently contained different ring types for 2005 including missing, normal, and dark rings.

The 2005 rings for trees at JAL were very narrow (average width = 0.45 mm) and contained both earlywood and latewood (Fig. 5c). The widest 2005 rings of Aleppo pine in this study were formed at GUA (average width = 1.7 mm). Most of the GUA 2005 rings contained an intra-annual density fluctuation (IADF) due to cambial reactivation in September/October (Fig. 5c).
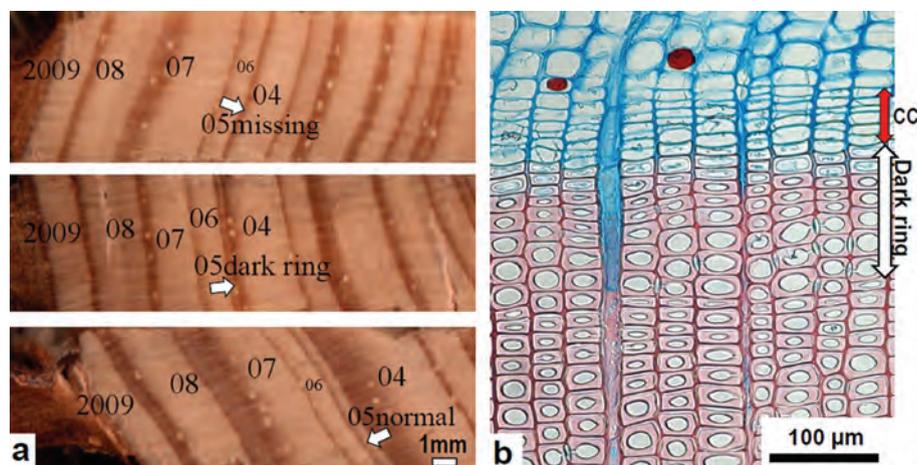

Figure 7. *Pinus halepensis* from MAI: a) Increment cores collected in 2009 (bark left) to show the 2005 (05) ring as missing (top), as a dark ring (middle), and normal (bottom); b) Stained cross section of sample collected in 2005 with the dark ring of latewood beneath the cambium (CC).

DISCUSSION

Detailed micro-anatomical examination coupled with retrospective dendrochronological analysis provided insight into the relationship of the tree-ring record of Aleppo pine to climate in southeastern Spain. The vascular cambium of trees at MAI did not increase in cell (CC) number per radial file during the 2005 growing season for Aleppo pine. Such an increase in CC number did occur at the comparison sites of GUA and JAL. The retention of the dormant characteristic of a thin cambium during the usual growing season at MAI was associated with a low level of cambial productivity, few new xylem cells, and no typical mature earlywood tracheids produced in 2005. The retrospective dendrochronological analysis showed a locally absent or missing 2005 ring in 50% of the cores collected at MAI.

The findings of PC and SW cells undergoing differentiation in anatomical samples collected in 2005 and the missing 2005 ring in some dendrochronological samples may be reconciled in two ways. First, the apparent discrepancy may simply reflect variable degrees of cambial activity around the stem circumference. Second, the few xylem cells produced by some radial files of the vascular cambium may have differentiated into tracheids with the appearance of latewood without production of earlywood. The thin layer of apparent latewood in the 2005 ring may have been mistaken for latewood of the 2004 growth ring.

We propose the term "dark ring" for rings which are dark in appearance in reflected light and which consist primarily or exclusively of latewood-like cells. Although visually similar to latewood with respect to in dendrochronology samples, dark ring cells may not meet the formal (and variously interpreted) criteria for latewood based on the proportion of tracheid thickness and lumen diameter (Denne 1988; Antony *et al.* 2012).

The new term is in contrast with the term "light ring" which is applied to light-coloured anomalous rings with one or a very few layers of latewood cells. Light rings are usually associated with subarctic environments in response to unusually shortened growing seasons (Filion *et al.* 1986; Liang & Eckstein 2006; Tardif *et al.* 2011). The dark rings at MAI contained tracheids with relatively small radial dimensions and thick cell walls. We suspect that they were very likely formed in other years as well but had not been identified as a distinct type of annual ring.

Latewood formation is associated with reduced radial expansion of tracheids, mediated in part by the gradient of indole-3-acetic acid (IAA) across the differentiating xylem (Uggla *et al.* 1996) and cell walls thickening which depends on concentration gradients of the carbohydrates and the capacity of tissues to use them in metabolic processes (Uggla *et al.* 2001). Reduced water potential during drought periods and the resulting reduction of turgor pressure (Hölttä *et al.* 2010) would also likely limit cell expansion along the radial plane. Reduced radial expansion reduces vulnerability to cavitation (Steppe *et al.* 2015). The structure of dark rings suggests a structural compromise that reduces risk of cavitation through reduced cross-sectional area of conducting tracheids while providing greater mechanical support to tracheids under conditions of increasingly negative pressure in the xylem water columns (Hacke *et al.* 2001; Jyske & Hölttä 2015).

The occurrence of more than one type (normal, missing, and dark) of 2005 ring in duplicate cores from individual trees demonstrated that the missing ring in one sample may more accurately be considered as "locally absent" as previously observed (Novak *et al.* 2011). These findings indicate that processes of cell division and differentiation are sensitive to small differences in environmental and biological constraints. In this research, these anomalies are associated with reduced precipitation and drought.

Missing 2005 rings were identified only at MAI. At the comparison sites, the late onset and early cessation of cambial activity in JAL resulted in narrow rings with both earlywood and narrow latewood. Interestingly, the widest rings of 2005 were found at GUA which overall is a warmer and drier location. However, winter precipitation and favourable temperature at GUA supported the early onset of cambial cell division in February and the development of well-defined earlywood. Dry conditions in July resulted in maturation of latewood tracheids. Unusually high precipitation in August through September resulted in a second period of earlywood formation and a clearly visible intra-annual density fluctuation or false ring (De Luis *et al.* 2011a). The relationship of missing rings to drought is supported by earlier research (*e. g.*, Rozas *et al.* 2011; Khishigjargal *et al.* 2014; De Micco *et al.* 2016). However, our study also suggests that micro-site conditions can play an important role for missing rings occurrence.

CONCLUSION

The integrated wood formation and dendrochronological analysis links missing ring formation (the absence of an annual ring) to the extreme drought of 2005 in southeastern Spain. Missing rings were associated with locally inhibited cambial cell production as observed on micro-anatomical samples. Distinct dark rings (the term first applied here to rings which appeared to contain only latewood-like cells) in other dendrochronological samples also provided an anatomical marker of extreme drought. Both missing rings and dark rings are markers that may be incorporated into dendroclimatic investigations of conifers in the environmentally sensitive Mediterranean Basin.

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