



Tree Physiology 37, 523–535
doi:10.1093/treephys/tpx031



Research paper

Isotope signals and anatomical features in tree rings suggest a role for hydraulic strategies in diffuse drought-induced die-back of *Pinus nigra*

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Received June 14, 2016; accepted March 8, 2017; published online March 17, 2017; handling Editor Roberto Tognetti

The 2003 and 2012 summer seasons were among the warmest and driest of the last 200 years over southeastern Europe, and in particular in the Karst region (northeastern Italy). Starting from winter-spring 2013, several black pines (*Pinus nigra* J.F. Arnold) suffered crown die-back. Declining trees occurred nearby individuals with no signs of die-back, raising hypotheses about the occurrence of individual-specific hydraulic strategies underlying different responses to extreme drought. We investigated possible processes driving black pine decline by dendrochronological and wood anatomical measurements, coupled with analysis of tree-ring carbon ($\delta^{13}\text{C}$) and oxygen ($\delta^{18}\text{O}$) isotopic composition in healthy trees (H) and trees suffering die-back (D). Die-back trees showed higher growth rates than H trees at the beginning of the last century, but suffered important growth reduction following the dry summers in 2003 and 2012. After the 2012 drought, D trees produced tracheids with larger diameter and greater vulnerability to implosion than H ones. Healthy trees had significantly higher wood $\delta^{13}\text{C}$ than D trees, reflecting higher water-use efficiency for the surviving trees, i.e., less water transpired per unit carbon gain, which could be related to lower stomatal conductance and a more conservative use of water. Relatively high $\delta^{18}\text{O}$ for D trees indicates that they were strongly dependent on shallow water sources, or that they sustained higher transpiration rates than H trees. Our results suggest that H trees adopted a more conservative water-use strategy under drought stress compared with D trees. We speculate that this diversity might have a genotypic basis, but other possible explanations, like different rooting depth, cannot be ruled out.

Keywords: carbon isotope, cell wall thickness, drought, Karst, oxygen isotope, tracheid lumen area, tree-ring width.

Introduction

The ongoing global warming is expected to affect terrestrial ecosystems by influencing the frequency, severity and spatial distribution of extreme weather events (Couchou and Rahmstorf 2012). In particular, droughts and heat waves are expected to intensify in the current century, as a result of reduced precipitation or increased evapotranspiration (Orlowski and Seneviratne 2012), but large uncertainties exist on how forests will respond to such changes. Temporal and spatial patterns of tree mortality rates recorded in some areas of the globe suggest that forest decline is increasing at pace, with increased frequency and

severity of heat waves and droughts; however, the physiological and ecological mechanisms, and structural adjustments, underlying these processes, as well as the main environmental drivers, are still poorly understood (Carnicer et al. 2011, Gaylord et al. 2015, Steinkamp and Hickler 2015).

Two non-exclusive mechanisms are likely to play a major role in drought-induced tree die-back, i.e., hydraulic failure and carbon starvation (McDowell et al. 2008, Anderegg et al. 2012, 2015). Severe drought and heat waves can impact trees via xylem embolism formation leading to desiccation (Rennenberg et al. 2006, Nardini et al. 2013). Also, increasing temperatures

raise atmospheric vapour pressure deficit, thus forcing water loss by transpiration. Plants react to increased evaporative demand by a continuum of stomatal responses (Klein 2014). These range from an 'isohydric' strategy, with stomata closing at specific water-potential thresholds to minimize further transpiration and limiting the xylem pressure drop, to an 'aniso-hydric' behaviour, where stomatal closure is delayed to allow continued carbon gain even at high transpiration rates, leading to substantial xylem tension build-up (McDowell et al. 2008, Tombesi et al. 2014). Both strategies have important drawbacks. In fact, the isohydric response can lead to carbon starvation, as stomatal closure shuts down photosynthesis while respiration continues to deplete carbohydrate reserves (Klein et al. 2011). On the other hand, the aniso-hydric response maximizes carbon gain while increasing the risk of xylem embolism and hydraulic failure.

Anomalous tree mortality events have been recently reported in European countries like France (Breda et al. 2006), Spain (Martínez-Vilalta and Piñol 2002) and Norway (Solberg 2004). In Italy, Vacchiano et al. (2012) indicated drought stress as a factor inducing decline in *Pinus sylvestris* L. populations, while Nardini et al. (2013) reported that the severe 2012 summer drought resulted in extensive crown desiccation of several tree species in the Classical Karst area (Trieste, northeastern Italy). In particular, throughout the Classical Karst, a remarkably high number of black pines (*Pinus nigra* J.F. Arnold) suffering die-back has been observed following the 2012 summer drought. Interestingly, declining trees were found to occur nearby conspecific individuals with no signs of die-back or decline, raising interesting questions on individual-specific functional traits underlying different responses to extreme drought. Indeed, previous studies have shown that members of the family Pinaceae, and particularly *Pinus* species, are more vulnerable to drought-induced mortality than other conifers (Martínez-Vilalta et al. 2004). In particular, *P. nigra* is a relatively drought-sensitive species (Martín-Benito et al. 2008) and likely vulnerable to the recently observed increase in temperature and decrease in precipitation.

Tree-ring width is a well-established proxy for tree performance over the entire growing season that can be used to infer past growth conditions and tree responses even centuries back in time (Cherubini et al. 2003, Fonti et al. 2010, Camarero et al. 2015). Xylem anatomical features within tree rings such as tracheid lumen area and cell wall thickness are particularly interesting because of their direct link to hydraulics and carbon allocation, and because these features are affected by internal and external factors throughout the growing season. Drought stress might be associated with the formation of more resistant tracheids, having narrower lumina and thicker walls (Hacke et al. 2015). However, also the opposite pattern could be observed to optimize the carbon-per-conduit costs under drought by increasing conduit diameter while decreasing cell wall thickness (Eilmann et al. 2009). Anatomical properties can thus give detailed mechanistic insight into tree functioning in principle over time frames as long as when

analysing tree-ring width (Pellizzari et al. 2016). Similarly, stable isotopes are useful tools to understand plant–environment interactions, with special reference to climate. In fact, climate strongly influences the processes and pathways through which carbon and oxygen are assimilated into organic matter. The $\delta^{13}\text{C}$ of biomass accumulated in tree rings is a useful proxy for the ratio of stomatal conductance to photosynthetic rates and thus water-use efficiency (McCarroll and Loader 2004, Cernusak et al. 2013). On the other hand, $\delta^{18}\text{O}$ is often correlated with water sources accessed by the root system (Burk and Stuiver 1981), as well as with transpiration rates, thus representing a proxy for rooting depth or for changes in the evaporative demand and plant water loss (Saurer et al. 1997, Leonelli et al. 2014). Hence, the combined analysis of $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ in tree-rings, coupled to wood anatomical features, can potentially highlight structural and functional adjustments of trees to fluctuations in water availability and climatic features (Rossi et al. 2013, Cernusak and English 2015). In fact, stable isotope variations have been successfully applied to better understand the relationship between growth and gas-exchange properties in tree-mortality studies (McDowell et al. 2010, Hentschel et al. 2014).

In this study, we aimed at obtaining information on black pine mortality processes in the Classical Karst through dendrochronological and wood anatomical analyses, coupled with analysis of tree-ring carbon ($\delta^{13}\text{C}$) and oxygen ($\delta^{18}\text{O}$) isotopic composition in healthy and declining trees. We focused on two recent drought events that occurred in the area, i.e., the summer seasons 2003 and 2012. These periods were among the warmest and driest of the last 200 years in SE Europe, including Italy (Schär et al. 2004, Ciais et al. 2005, Sippel and Otto 2014, Orth et al. 2016). In particular, in our study area summer temperature anomalies were about +3.5 °C and +2 °C in 2003 and 2012, respectively. Moreover, summer precipitation anomalies averaged –30 and –45% in 2003 and 2012, respectively (data from www.isac.cnr.it). Our purpose was to verify whether the ongoing crown die-back of black pine in the Classical Karst was actually triggered by these recent extreme climatic events. Our main focus was the comparison of two groups of trees from the same site. We expected declining individuals to be more sensitive to drought in terms of: (i) lower growth rate, (ii) higher vulnerability to embolism as inferred from xylem anatomical features and (iii) higher water-use efficiency due to stomatal closure as a safety mechanism to avoid hydraulic failure, in the frame work of an isohydric strategy.

Materials and methods

Study area and climate

The study was conducted on wood cores collected from *P. nigra* trees in a plantation (Bazzoni wood: 45°37'50"N; 13°51'08"E; 385 m above sea level (a.s.l.)) located in the Classical Karst area near the city of Trieste (Italy). The plantation was settled between late 1800 and early 1900 (Poldini 1989, Sfregola 2017), and the stand is currently undergoing partial regrowth of

natural vegetation in the understory, with scattered individuals of *Quercus* sp., *Fraxinus ornus* and *Acer campestre*. The Classical Karst is a vast morphological unit dominated by carbonate rocks and characterized by typical dissolution features (Cucchi et al. 1987). Upper soils are poorly developed, but complex fissures and cave systems offer spaces where roots can penetrate, often filled with clay soils representing potentially important water reservoirs for plants (Nardini et al. 2016). The climate in the area is transitional between oceanic-Mediterranean and prealpine-continental type, with rainy winters and dry summers (Poldini 1989). The years 2003 and 2012 were dryer and warmer compared with the mean of the decade 1993–2013, especially during the summer months (Figure 1). The annual mean air temperature at the Sgonico weather station (45°44'16"N; 13°45'07"E; 268 m a.s.l.; ~14 km from our study area; data from www.osmer.fvg.it) calculated for the period 1993–2013 is 12.8 °C. The years 2003 and 2012 showed the highest number of days with maximum air temperature above 30 °C, respectively 66 and 53. The mean annual precipitation was 1367 mm, with 2003 as the driest year (974 mm), while in 2012 the cumulative rainfall was comparably high with 1199 mm, but summer precipitation was 48% less than in the long-term average.

Collection of samples and tree-ring width analyses

Wood core samples were collected during the winter growth dormancy on 21 February 2014, after a visual assessment of the crown status of ~300 adult trees with an estimated age of ~50 years. Among these, 30 trees of similar size were selected

and classified in two groups, H (healthy) and D (die-back) (15 individuals each). Healthy trees did not show any sign of crown die-back, whereas D ones showed desiccation in ~50% of the crown, as visually estimated by four different observers to reduce possible subjective errors (Schomaker et al. 2007). The diameter at breast height (DBH) was measured for each individual. Two cores were taken at breast height from each tree (west and east directions) with an increment borer (diameter 5 mm). The cores were transported to the laboratory and air dried. The surface of the cores was levelled with a microtome (WSL-Core-Microtome, Schenkung Dapples, Zürich, Switzerland). Cores were observed under a stereomicroscope (Wild M3Z Leica, Wetzlar, Germany), and each tree ring was dated, i.e., assigned a calendar year. Tree-ring width was measured to the nearest 0.01 mm using a LINTAB digital positioning table and the software TSAPWin (RINNTECH, Heidelberg, Germany). Tree-ring series were visually cross-dated and compared using standard dendrochronological techniques (Stokes and Smiley 1968). The cross-dating accuracy was then checked using the program COFECHA (Holmes 1983, Grissino-Mayer 2001). Tree-ring chronologies were not standardized in order to maintain eventual climatic signals as strong as possible (Briffa et al. 1998, Esper et al. 2002).

Quantification of wood anatomical features

Anatomical measurements were performed on a subset of six trees (three per group). The selected trees had the ring width most similar to the mean series of the respective group. Tree-ring analysis revealed that sampled trees belonged to two different age classes, but the percentage of younger versus older trees was not different in H and D groups (see below). Hence, the criterion used to select the subset was similarity of ring width to the mean series of the respective groups, resulting in the identification of three younger trees from the D group, and one younger and two older individuals from the H group. From the cores of the six selected trees, only the portion including rings produced from 2000 to 2013 was used to obtain thin (18 µm) sections, using a microtome (WSL-Lab-Microtome, modified Reichert-type, Schenkung Dapples). Microsections were stained, dehydrated and embedded. Several overlapping images covering the entire sections were taken with a digital camera (EOS 650D Canon, Tokyo, Japan) connected to a light microscope (BX41 OLYMPUS, Tokyo, Japan) at a magnification of $\times 100$, corresponding to a resolution of $2.36 \text{ pixel } \mu\text{m}^{-1}$. A single image of each section was obtained using the merging software Autopano Pro (Kolor SARL, Francin, France). From the whole image of each section, a strip of ~1.4 mm width was cropped, covering the years 2002–04 and 2011–13 to include the periods before and after the most recent summer droughts (2003 and 2012).

Xylem anatomical features of all tracheid cells ($n = 66,885$) were measured using the specialized image-analysis tool ROXAS v2.0 (von Arx and Carrer 2014). To estimate water transport efficiency, the 95th percentile of the conduit lumen

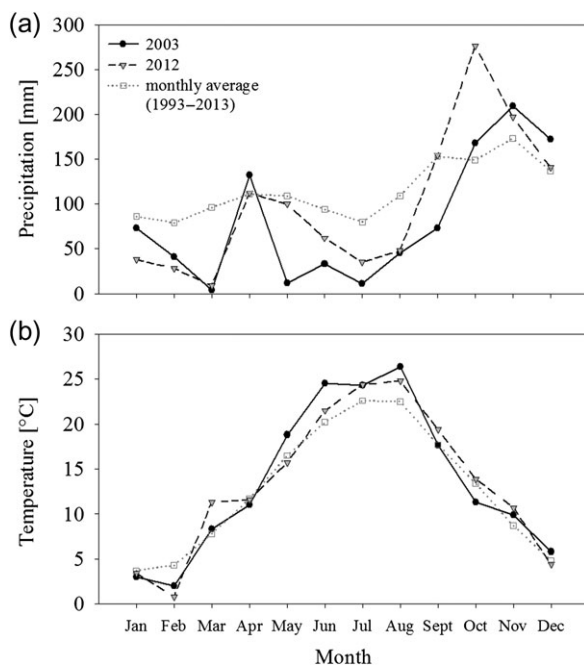


Figure 1. Monthly means of cumulative precipitations (a) and temperatures (b) in the period 1993–2013 compared with the years 2003 and 2012 (data from OSMER Sgonico weather station).

area distribution (CA; corresponding to the 5% of the widest, i.e., most efficient, tracheids) and the theoretical hydraulic conductivity per ring (KH; based on Hagen–Poiseuille's law) were calculated. KH was obtained by multiplying the area-specific hydraulic conductivity by ring width to reduce geometric effects of different stem diameters. As a proxy of carbon allocation, we calculated for each ring the 5th and 95th percentile of the cell wall thickness distribution. Since the thinnest tracheid walls typically occur in the earlywood and the thickest tracheid walls in the latewood (Cuny et al. 2014), we hereafter refer to these extremes as earlywood (EWT) and latewood (LWT) cell wall thickness, respectively. Tracheids' mechanical resistance to implosion was estimated by calculating for each cell the 'bending resistance index' $(t/b)^2$ (BEND) (Hacke et al. 2001). The value of the 5th percentile of each ring corresponding to the less resistant end of the distribution was used for analyses to consider the weak point. The intra-annual position where the average Mork index attained values >1 was used as the transition between earlywood and latewood (Denne 1988), and earlywood and latewood width were calculated to estimate growth in two distinct intra-annual periods.

Isotopic analysis

Analysis of carbon and oxygen stable isotopes was performed on the same samples used for anatomical measurements. Although we are aware that carbon incorporated in the earlywood could originate from remobilized starch (Gessler 2011, Offermann et al. 2011), the whole ring was used in the analyses to obtain a sufficient amount of wood, and because early and latewood isotope ratios were previously shown to be rather similar for conifers (Kress et al. 2009). Wood cores were carefully separated into sections corresponding to specific growth years using a razor blade under a stereomicroscope. In the case of rings produced in the years 1970–99, for each year the six samples from both group of trees were pooled together. For the last 14 years (2000–13), the rings were collected separately from each sample. For each tree, the rings of the two cores formed in the same year were pooled together.

Wood was ground with a Mixer Mill MM2000 (Retsch, Hann, Germany) and cellulose was extracted according to Battipaglia et al. (2014). The cellulose was weighed with a MX5 microbalance (Mettler Toledo, Greifensee, Switzerland), and analysed using a Continuous Flow Isotopic Ratio Mass Spectrometer (CF-IRMS) based on an elemental analyser (Euro EA, HEKAtech GmbH, Wegberg, Germany), coupled with a mass spectrometer (IRMS Delta V Advantage, Thermo Fisher Scientific, Waltham, MA, USA) via a variable open split interface (Conflo III, Finnigan, Germany). For $\delta^{13}\text{C}$ analysis, samples were combusted in an oxygen atmosphere, while material for $\delta^{18}\text{O}$ analysis was decomposed to CO by thermal pyrolysis at 1080 °C (Saurer et al. 1998). All results were expressed as ‰ relative to the standard (VPDB for $\delta^{13}\text{C}$ and VSMOW for $\delta^{18}\text{O}$).

A decline in the raw $\delta^{13}\text{C}$ chronology, mainly in the 20th century, is usually observed and attributed to the progressive decrease of atmospheric $\delta^{13}\text{C}$ due to combustion of fossil fuels (Cullen and Grierson 2007). This trend was removed in the carbon isotope chronology using the annual records of past atmospheric $\delta^{13}\text{C}$ obtained from ice core and atmospheric data (Leuenberger 2007).

Climate correlations

Ring width and isotopic ratios were correlated with climatic data (cumulative monthly precipitation, mean, maximum and minimum temperature) obtained from KNMI Climate Explorer (using minimal fraction of valid points 30.00, E-OBS analyses v10.0, cutting out region longitude = 13.750–14.000, latitude = 45.500–45.750) (Trouet and Van Oldenborgh 2013). The time period covered by climatic data goes from 1950 to 2013.

Statistical analyses

The data were analysed for normality of the distribution and the equality of variance, and the significance of annual differences between H and D was checked using Student's *t*-test or Mann–Whitney *U* test. The statistical significance of correlations was tested using the Pearson's Product Moment Correlation. To investigate potential responses of anatomical features to the 2003 and 2012 droughts, the raw values were standardized by dividing them by the mean of the respective individual and drought period (2002–04 and 2011–13, respectively), thus removing tree-specific offsets due to tree-height-related trends (Carrer et al. 2015). For each anatomical variable, linear mixed-effect models with year, tree group (H and D), the interaction between the two and tree ID as a random factor to account for the repeated measurements (Crawley 2007) were compared with identical general linear models, apart from the random term using AICc (Akaike Information Criterion) (Zuur et al. 2009). In all cases, the general linear models proved to be more appropriate and were therefore selected. The initial general linear models were simplified using dredging techniques based on AICc to obtain the optimal model. Normality and homoscedasticity requirements were checked using Shapiro–Wilk's and Bartlett's test, respectively. These statistical analyses were performed in R version 3.1.0 including the package 'MuMIn'.

Results

Growth responses to long-term climate variability and drought

Die-back trees showed ring-width patterns overall similar to healthy (H) ones, with the exception of the juvenile period (1915–25) and of 2013, when highly significant differences ($P < 0.001$) emerged between the two chronologies (Figure 2). Correlation coefficients between the single-core series and respective means were all significant ($P \leq 0.05$), with $P \leq 0.001$

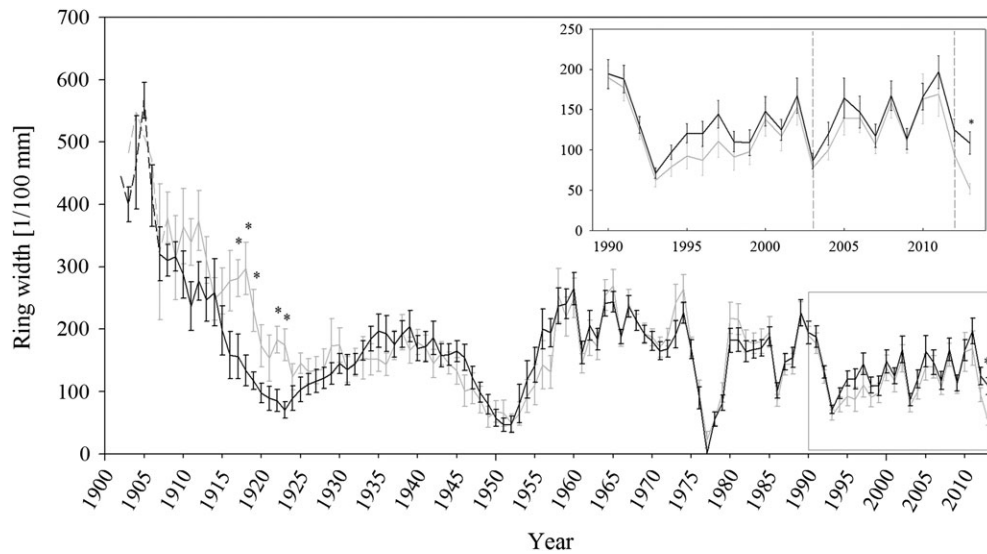


Figure 2. Mean raw ring-width chronology of the healthy trees (black) and those suffering die-back (grey). Bars indicate \pm mean standard error. Broken lines signify five trees or fewer. Significant differences ($P \leq 0.05$) have an asterisk (*). The inset shows the period from 1990 to 2013 in more detail. Dashed lines highlight the drought years investigated.

for 55 cores out of 60. Although the visual assessment at the time of sampling did not allow us to distinguish different age classes, the innermost ring distribution revealed the existence, in samples from both H and D trees, of two different groups: one group comprised old trees with an estimated age of ~ 100 years, and a second one included younger trees, ~ 50 years old. This difference was not apparent at the time of sampling, as the two groups have very similar size. In fact, DBH was 35.8 cm (SD = 2.5 cm) and 28.8 cm (SD = 3.0 cm) in old and young trees, respectively, and this difference was not statistically significant ($P = 0.22$). The percentage of H and D trees was similar in the two age classes, with nine old and six young individuals among H trees, and seven old and eight young among D trees. Moreover, young and old trees shared the same pattern of growth shown in the main chronologies. Comparing ring-width chronologies built using trees of different age may bias the results because of possible age effects induced by physiological and geometrical factors. However, we mainly focused our analysis on the ring width of older and younger trees during the past two decades only, more exactly from 2003 onward. During this specific time interval, both the older and the younger tree-ring width chronologies are not affected by any age trend anymore. In fact, an age trend is generally apparent in ring-width chronologies only in the first three-four decades after germination in *P. nigra*.

After testing all individual months as well as different groups of months (see Supplementary Data at [Tree Physiology Online](http://www.treephys.oxfordjournals.org)), June rainfall as well as the cumulative rainfall falling from April to July turned out to be important climatic factors influencing ring width (Figure 3a). The correlations were positive and significant for all trees, but H trees showed a stronger response ($r = 0.354$, $P = 0.004$) than D ones ($r = 0.277$, $P = 0.028$).

Including also precipitations of August and September, the correlations remained positive and significant for both groups. Overall, growth rates of H trees had a stronger correlation with summer precipitation than those of D trees. The mean of maximum temperatures recorded from April to July correlated negatively with mean ring width (MRW) in both groups, but significantly only in the case of D trees ($r = -0.327$, $P = 0.008$) (Figure 3b).

The lowest growth rates were observed in the driest years (2003 and 2012). After the 2003 drought, secondary growth recovered in both groups, but faster in H than in D trees. However, after summer 2012, D trees did not recover at all. In fact, in 2013 the rings in both groups were even narrower than in the previous year, but the decrease in ring width from 2012 to 2013 was significant only in D trees ($P = 0.003$).

Growth responses of study trees were investigated in terms of ring width, which was preferred to basal area increment because only wood cores were available, and calculating basal area increment without access to whole cross sections would have introduced a non-negligible error in the measure.

Anatomical responses to drought

The 2012 summer drought produced stronger impacts than that of 2003 on the anatomical features investigated (Table 1). Both drought events induced a significant decrease of the MRW. However, MRW partly recovered to pre-drought values after the first 2003 event, while it declined further after the second severe drought in 2012 (Figure 4a, Table 1). These patterns were similar in H and D trees, but in 2013 D trees produced significantly narrower rings than H ones. Responses in ring width of the subset trees were similar to those for the entire data set

(Figure 4a, Table 1), thus confirming that the six selected trees were representative for the entire data set. Earlywood width (EWW, Figure 4b) showed patterns of variation similar to those

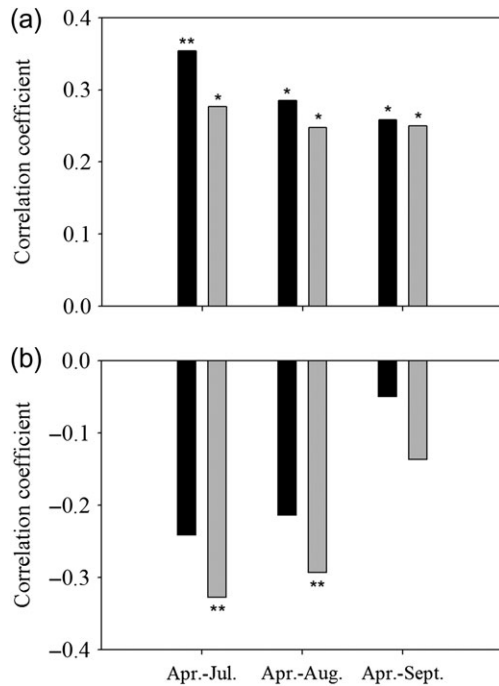


Figure 3. Pearson's correlation coefficients between MRW, of either healthy trees (black) or trees suffering die-back (grey) (period 1950–2013), and cumulative precipitation (a) and maximum temperature (b) during April–July, April–August and April–September. * $P \leq 0.05$, ** $P \leq 0.01$.

recorded for MRW on the first drought period, but in the second drought event D trees produced relatively more earlywood than H trees. In 2013, EWW was smaller in D than in H trees due to a significant reduction recorded in D plants.

We found no significant responses to the first drought event in terms of lumen area of the widest cells (CA, Figure 5a, Table 1), but significant responses occurred in the second one, with measurable differences between H and D trees. In particular, CA increased only in D trees in 2012 and then remained high, as H trees reached the same CA level in 2013. In the first drought period, the theoretical ring conductivity (KH) decreased and only partially recovered in the following year in both groups (Figure 5b). In the second drought period, KH decreased only in H trees ($P = 0.004$) and then remained low. In D trees, KH only marginally decreased from 2011 to 2012, but significantly from 2012 to 2013.

Changes in EWT showed no significant pattern in response to the first drought period, while in the second one significantly thinner walls were recorded, with no recovery in the following year in both groups (Figure 6a, Table 1). We observed a very strong and consistent decrease in LWT in 2003, with partial recovery 1 year later (Figure 6b, Table 1). Latewood cell wall thickness decreased also in the second drought period, but in this case, as for EWT, no recovery was recorded 1 year later. The bending resistance index (BEND) increased in the first drought year and returned to pre-drought values 1 year later (Figure 6c, Table 1). In the second drought period, BEND decreased in both groups, but more strongly in D trees. While anatomical responses in trees are often temporally closely (weeks to months) connected

Table 1. Results of linear models (estimates \pm SE) of the analysed anatomical parameters, expressed as relative values.

Variables	MRW	EWW	CA	KH	LWT	EWT	BEND
2003 drought							
Intercept	1.35 \pm 0.06	1.20 \pm 0.05	–	1.26 \pm 0.06	1.19 \pm 0.02	–	0.89 \pm 0.05
2002	0 ^a	0 ^a	–	0 ^a	0 ^a	–	0 ^a
2003	–0.75 \pm 0.08 ^c	–0.40 \pm 0.07 ^c	–	–0.55 \pm 0.08 ^c	–0.41 \pm 0.02 ^c	–	0.28 \pm 0.07 ^b
2004	–0.31 \pm 0.08 ^b	–0.19 \pm 0.07 ^b	–	–0.21 \pm 0.08 ^b	–0.15 \pm 0.22 ^b	–	0.06 \pm 0.07 ^a
Healthy	–	–	–	–	–	–	–
Desiccated	–	–	–	–	–	–	–
Year: Status	–	–	–	–	–	–	–
Model perform.	$R^2_{\text{adj}} = 0.83^{***}$	$R^2_{\text{adj}} = 0.67^{***}$	ns	$R^2_{\text{adj}} = 0.72^{***}$	$R^2_{\text{adj}} = 0.95^{***}$	ns	$R^2_{\text{adj}} = 0.45^{**}$
2012 drought							
Intercept	1.61 \pm 0.07	1.31 \pm 0.10	0.94 \pm 0.03	1.41 \pm 0.07	1.18 \pm 0.04	1.15 \pm 0.03	1.21 \pm 0.08
2011	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a
2012	–0.78 \pm 0.09 ^b	–0.51 \pm 0.14 ^b	0.02 \pm 0.04 ^a	–0.65 \pm 0.11 ^b	–0.25 \pm 0.05 ^b	–0.23 \pm 0.04 ^b	–0.20 \pm 0.11 ^a
2013	–1.08 \pm 0.09 ^c	–0.42 \pm 0.14 ^b	0.15 \pm 0.04 ^b	–0.57 \pm 0.11 ^b	–0.29 \pm 0.05 ^b	–0.23 \pm 0.04 ^b	–0.42 \pm 0.11 ^b
Healthy	–	0 ^a	0 ^a	0 ^a	–	–	0 ^a
Desiccated	–	–0.13 \pm 0.14 ^a	–0.08 \pm 0.04 ^a	–0.09 \pm 0.11 ^a	–	–	0.25 \pm 0.11 ^b
Year 2011: Desicc	–	0 ^a	0 ^a	0 ^a	–	–	0 ^a
Year 2012: Desicc	–	0.61 \pm 0.20 ^b	0.23 \pm 0.06 ^b	0.53 \pm 0.15 ^b	–	–	–0.49 \pm 0.16 ^b
Year 2013: Desicc	–	–0.23 \pm 0.20 ^a	0.01 \pm 0.06 ^a	–0.25 \pm 0.15 ^a	–	–	–0.10 \pm 0.16 ^a
Model perform.	$R^2_{\text{adj}} = 0.89^{***}$	$R^2_{\text{adj}} = 0.73^{***}$	$R^2_{\text{adj}} = 0.70^{***}$	$R^2_{\text{adj}} = 0.87^{***}$	$R^2_{\text{adj}} = 0.68^{***}$	$R^2_{\text{adj}} = 0.72^{***}$	$R^2_{\text{adj}} = 0.78^{***}$

Significance of models at 0.01 and 0.001 probability levels are marked with ** and ***, respectively. Different superscripts indicate different levels of significance. Marginally significant superscripts are shown in italics. Non-significant superscripts are shown in underline.

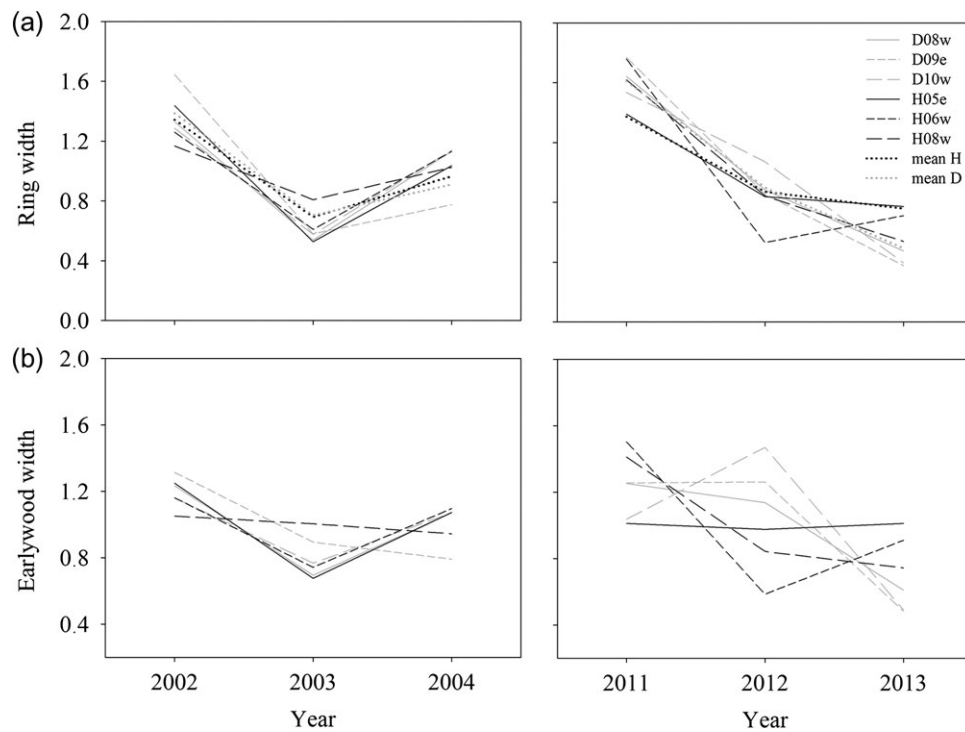


Figure 4. Relative values of ring width (a) and EWW (b) of trees healthy (H, black) or suffering die-back (D, grey) during the two drought periods analysed. Data from the same individual (see individual codes in the legend, where the number indicates the individual and w or e refers to the aspect where cores were collected) are represented in the same colour and line style. Means reported in Figure 5a (dotted lines) are derived from LINTAB analysis (see text for details).

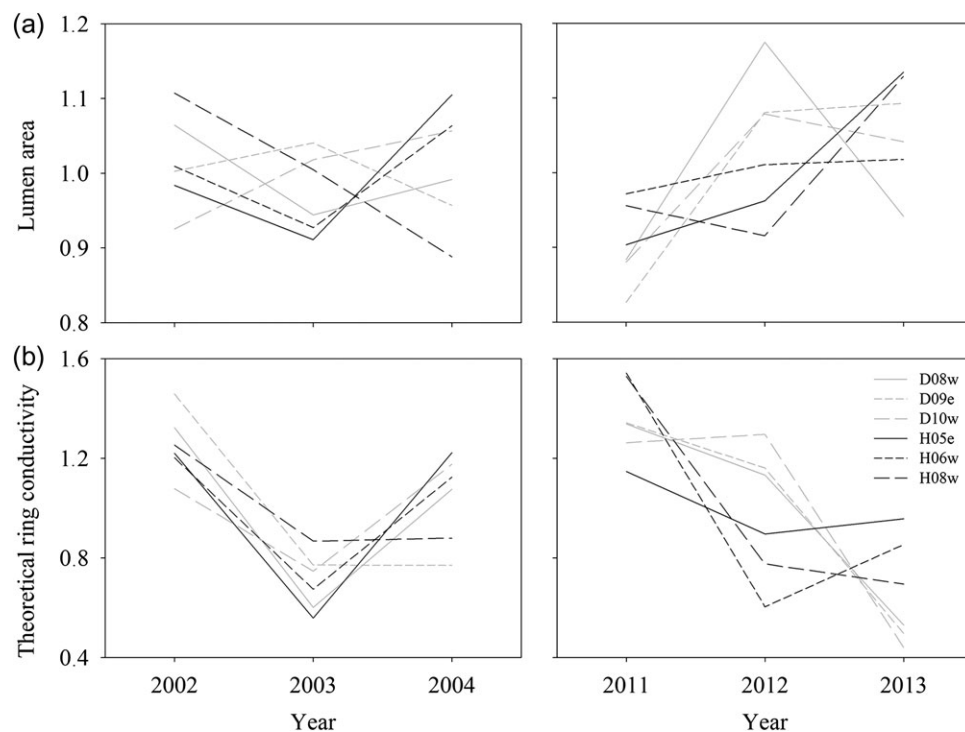


Figure 5. Relative values of lumen area of widest cell (a) and theoretical ring conductivity (b) of trees healthy (H, black) or suffering die-back (D, grey) during the two drought periods analysed. Data from the same individual (see individual codes in the legend, where the number indicates the individual and w or e refers to the aspect where cores were collected) are represented in the same colour and line style.

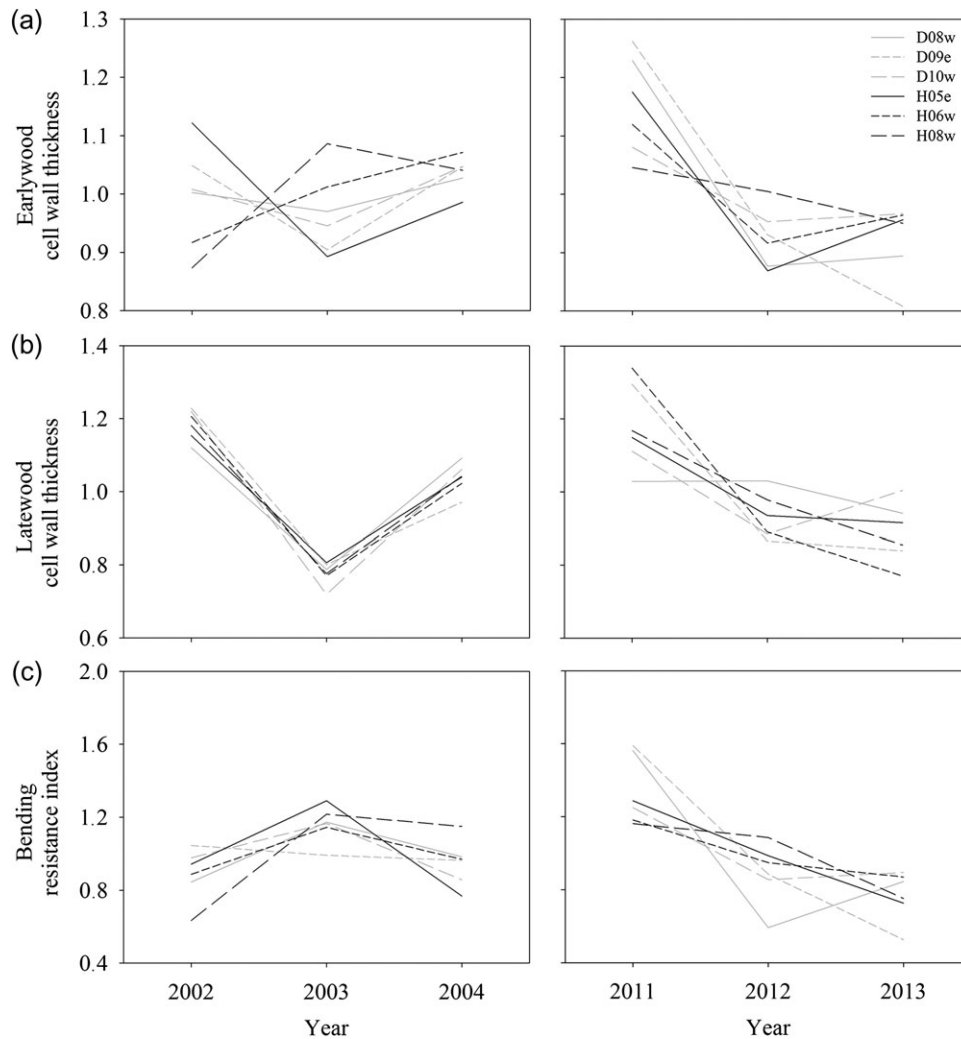


Figure 6. Relative values of EWT (a), LWT (b) and bending resistance index (c) of healthy trees (H, black) and of trees suffering die-back (D, grey) trees during the two drought periods analysed. Data from the same individual (see individual codes in the legend, where the number indicates the individual and w or e refers to the aspect where cores were collected) are represented in the same colour and line style.

to conditions during wood formation, there can also be lagged responses of one to several years. Therefore, longer term anatomical responses might be missed by the limited time period considered for these analyses.

Carbon and oxygen stable isotopes

Die-back trees had on average lower $\delta^{13}\text{C}$ and higher $\delta^{18}\text{O}$ values than H ones (Figure 7). The correlation between $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ was significant, both for H and D trees (respectively $r = 0.422$; $P = 0.005$, and $r = 0.500$; $P < 0.001$) (Figure 8). Mean values of $\delta^{13}\text{C}$ in H and D trees in the period 1970–2013 were -22.4 and -23.1‰ , while those of $\delta^{18}\text{O}$ were 33.4 and 33.8‰ , respectively. The most negative value of $\delta^{13}\text{C}$ for D trees was -27.2‰ (year 2013), while in H trees the most negative single value was -25.4‰ , recorded in 2012. The $\delta^{18}\text{O}$ was lower in H trees than in D ones. The lowest $\delta^{18}\text{O}$ values were recorded in wood produced in 2013 (31.7 and 32.1‰ in H and D trees, respectively).

Cumulative rainfall from October to March (winter precipitation) was negatively correlated to the $\delta^{18}\text{O}$ of H trees, but not of D ones ($r = -0.401$; $P = 0.009$). The $\delta^{13}\text{C}$ was positively and significantly correlated with the mean maximum temperature (T_{max}) during the period April–May in H trees, but not in D ones ($r = 0.394$; $P = 0.009$) (see Table S3 available as Supplementary Data at [Tree Physiology Online](#)).

Discussion

Growth history of D and H trees: prodigal versus provident strategies

Several different and independent proxies of trees' response to the two drought events in 2003 and 2012 converge to similar tentative conclusions, suggesting possible mechanistic explanations for tree decline recently observed in the Classical Karst. Air temperature increased over the century, possibly contributing to the progressive decline of these trees. The growth decline could

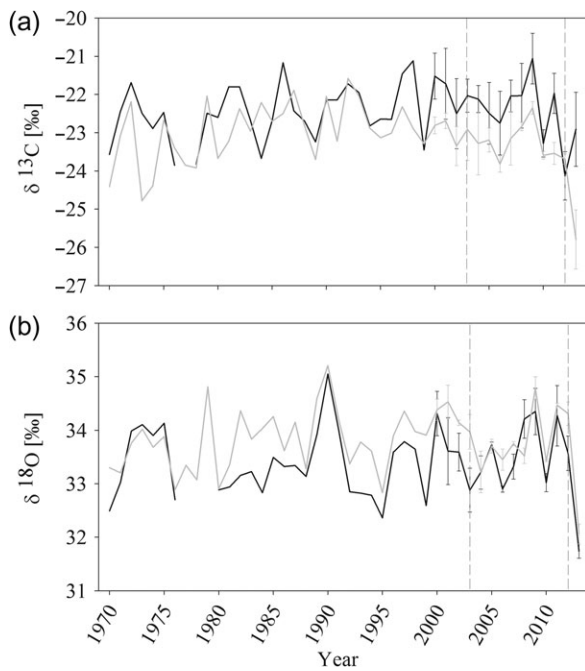


Figure 7. Single-year values of $\delta^{13}\text{C}$ (a) and $\delta^{18}\text{O}$ (b) in healthy trees (black) and trees suffering die-back (grey). Dashed lines highlight the drought years investigated.

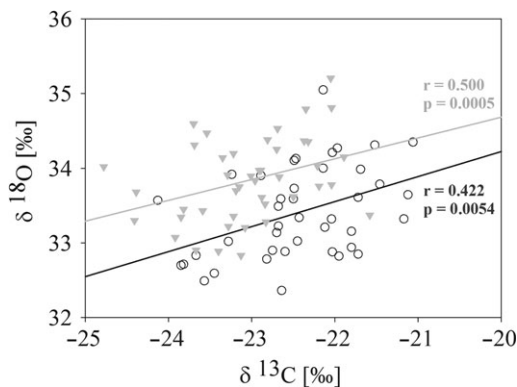


Figure 8. Correlation between carbon $\delta^{13}\text{C}$ and oxygen $\delta^{18}\text{O}$ isotope ratios in the healthy trees (black, empty dot) and in trees suffering die-back (grey, filled triangle).

be due to repeated stress events that made D trees more prone to die-back. Bigler et al. (2007) observed that several drought events occurring since 1864 coincided with decreased growth of fir, spruce and pine in a subalpine forest in the Rocky Mountains (Colorado, USA). Although single extreme drought years had a short-term and reversible effect on tree growth, multi-year drought-induced prolonged periods of growth decrease, enhancing long-term risk of tree death. Cavin et al. (2013) found that the lower growth rate of some *Fagus sylvatica* L. trees may have predisposed them to more pronounced drought response, in agreement with previous studies showing higher drought mortality for poorly growing canopy trees (Das

et al. 2007). Despite small differences in growth prior to the extreme drought event investigated, the *F. sylvatica* cohorts diverged in response to this event, with the most severely damaged trees taking a longer time interval to recover. Our data are in overall agreement with these results, as D trees returned, with delay, to growth rates typical of H trees after the 2003 drought.

A significant difference in growth rate at the beginning of the 20th century emerged between H and D older trees, with the latter growing faster than the former. Hentschel et al. (2014) explained differences between healthy and desiccated spruce trees in Norway on the basis of genetically determined growth rates and long-term resource use. They suggested the existence of a 'provident strategy' of non-symptomatic trees (with larger safety margins towards hydraulic failure, but also reduced growth under 'normal' years) and a 'prodigal strategy' in symptomatic trees (maximizing photosynthesis and growth while reducing hydraulic safety, to benefit from this strategy in years with high water supply but suffering hydraulic failure and growth decline during drought periods). A similar scenario apparently applies also to our study trees, as our data suggest occurrence of a provident strategy in H trees and a prodigal strategy in D ones.

Not surprisingly, the growth of both H and D trees was related to precipitation during the growing season. Moreover, both H and D trees responded to high temperatures by reductions in growth rate, but the response was more apparent in the latter, as a possible consequence of a hydraulic strategy leading to larger water loss by transpiration. The negative effect of high temperatures and drought on ring width is well documented in the literature, specifically in the case of *Pinus* sp.; as an example, Nabais et al. (2014) found a strong negative correlation between June and August temperatures and the growth of *Pinus pinaster* in Portugal.

Xylem anatomical features: the possible risks of compensation mechanisms

Variability in xylem anatomy is of particular interest when studying tree decline, because water transport plays an important role in plant performance and survival (Sperry 2003). Environmental conditions experienced by trees are likely to be recorded in the anatomical structure, but the quality and strength of the signal can vary according to climatic zone, season, species and anatomical feature considered (Fonti et al. 2010, Heres et al. 2014). In this study, the 2003 drought had a weaker effect on anatomical features than the second one occurring in 2012. This is in line with tree-ring width trends, showing a partial recovery after 2003 but not after 2012 droughts. The 2012 drought produced a continued detrimental effect on secondary growth, but this effect was stronger in D trees. While all trees modified their anatomical features during the second drought event, the responses were quite different between groups. In fact, in H trees ring width, latewood width and EWW all decreased under drought, and reduced growth rate persisted in the following

year. However, these trees apparently produced wider earlywood cells in 2013, thus reaching a higher total hydraulic efficiency (KH) than D trees. Healthy trees also maintained a higher tracheid implosion safety (BEND) in the driest years, thus possibly avoiding embolism. In contrast, D trees decreased the production of latewood but not of earlywood, and also formed wider but thin-walled tracheids, thus being possibly more vulnerable to embolism. This view is supported by Eilmann et al. (2011), who found wider cells with thinner walls in constantly drought-exposed *P. sylvestris* at Pfywald (Switzerland), and suggested that such a risky strategy responded to the need to optimize water uptake efficiency under carbon limitation because of reduced photosynthetic rates.

The largest earlywood cells are often most responsive to changes in water availability (Fonti and Garcia-Gonzalez 2004, Fonti et al. 2007, Martin-Benito et al. 2013, Fonti and Babushkina 2016). Lumen diameter could be considered as the final output of a trade-off between increasing efficiency and decreasing safety in water transport systems. Increases in the diameter of conductive elements lead to major gains in hydraulic conductivity (Domec and Gartner 2002), but may also imply higher vulnerability to embolism (Tyree and Zimmermann 2002). Even if there might be no direct link between lumen area and vulnerability to embolism, wide conduits tend to have by chance some larger pits more prone to air seeding ('rare-pit' hypothesis; Christman et al. 2009). In addition, hydraulic safety also relies on the mechanical strength of conduits, with thicker cell walls being associated with higher resistance to embolism formation. The response of the D trees to 2012 drought can be interpreted as a compensation mechanism to maximize water transport efficiency when water was limiting (Eilmann et al. 2009). The negative counterpart might reside in a higher risk of hydraulic failure. The consequences of this response might have become apparent in 2013, when D trees performed worse than H trees.

The drought response strategy of D trees likely exposed them to a higher risk of embolism or tracheid implosion. It is interesting to note that tracheid stability (BEND) showed contrasting immediate responses to drought in the two periods. In the first period, the increasing bending resistance of the earlywood cells increased the safety. The functional drought response in the first period was achieved by a combination of smaller earlywood cells and thicker earlywood cell walls, although individuals showed inconsistent patterns involving only one or both of these options, thus resulting in insignificant results when considering either of these anatomical features individually.

While the parameters associated with carbon pool size, secondary growth (ring width) and cell wall thickness, mostly returned to pre-drought levels after 2003, trees were apparently unable to recover after the second drought event (2012). This could suggest depletion or inability to mobilize carbon pools, due to damage that occurred to transport tissues (Woodruff 2014). The general water limitation at the study site, due to

karstic substrates, coupled to an increased frequency of harsh summer conditions, might have progressively reduced tree vigour. Latewood cell wall thickness is an indicator for carbon assimilation in summer, because latewood cell wall thickening mostly relies on recently assimilated carbon. The reduction in cell wall thickness observed in the driest years is therefore not surprising, but the persistent reduction even in the following year suggests that carbon pools and or carbon uptake capacity were still not fully restored, yet. We found no difference between H and D trees in terms of size of the largest cells in the first drought event. Lebourgeois (2000) suggested that climatic conditions affect more latewood growth than the size of the larger cells, which are commonly present in earlywood. Accordingly, Jyske et al. (2009) found no statistically significant differences in the average tracheid diameter between drought-treated and control Norway spruce trees. The wider earlywood cells observed during the second drought year (2012) in D trees are consistent with a prodigal/anisohydric strategy, exposing trees to a greater risk of hydraulic failure and decline, as also observed by Voltas et al. (2013) in Scots pine.

Carbon and oxygen isotopes in tree rings

Carbon isotope variability in plants is driven by isotope fractionations during uptake and assimilation of CO₂, where high stomatal conductance is usually related to lower water-use efficiency and higher inter-cellular CO₂ concentration resulting in relatively depleted $\delta^{13}\text{C}$ values (McCarroll and Loader 2004). Wood $\delta^{13}\text{C}$ in D trees was significantly lower than in H ones, suggesting that the former group delayed stomatal closure under drought while tolerating higher transpiration rates. This water-spending behaviour might lead to higher photosynthetic and growth rates under favourable environmental conditions as reflected in larger tree-ring growth of this tree group in earlier decades. On the other hand, such a strategy and increased water loss might represent a threat to plant survival in dry years. The higher $\delta^{13}\text{C}$ of H trees indicates an overall more conservative water use in this group (Scheidegger et al. 2000). Healthy trees had higher $\delta^{13}\text{C}$ values when the T_{max} was higher during the early growing season (April–May), suggesting that under warmer climate they effectively closed stomata to prevent excessive water loss. Our results show that $\delta^{13}\text{C}$ of D trees markedly decreased in 2013, indicating either increasing stomatal conductance (g_s) or decreasing photosynthetic rate (A), with the latter more likely due to the observed crown die-back leading to limitation of carbon supply. Similar results were obtained by Hentschel et al. (2014), who reported lower $\delta^{13}\text{C}$ values in declining *Picea abies* trees (interpreted as an increase in g_s), while healthy trees had low stomatal conductance, which likely prevented water loss during dry periods.

The oxygen isotope composition of tree rings is influenced strongly by soil water isotope changes that are driven by the isotope composition of precipitation and soil evaporation (Saurer et al. 1997). Climatic data indicated some influence of previous

spring/winter precipitation for $\delta^{18}\text{O}$, suggesting a stronger dependence of trees on this water (which has relatively low $\delta^{18}\text{O}$ signature compared with summer precipitation) in the year following the drought event (particularly in 2013). Such a long lag time of several months between growth and water source might suggest access to water sources with long recharge time intervals, like fissures and caves filled with clay deposits (Nardini et al. 2016). After winters characterized by heavy precipitations, the $\delta^{18}\text{O}$ of H trees was significantly lower than in D trees, indicating water uptake from these reserves in H plants, while D ones may have limited access to such soil pockets, fissures and caves (Treydte et al. 2014). Plants with deep roots can maintain more favourable water status and gas-exchange rates, thus maintaining stable or even increasing their non-structural carbohydrate content between spring and summer (Nardini et al. 2016), which can be used during periods of stress. The significant positive correlation between carbon and oxygen isotope variations indicates a limitation of photosynthesis by stomatal constraints for *P. nigra* at this site, but due to similar slopes of the regression line this analysis does not show a clear difference in gas-exchange response patterns between the groups.

Our results suggest that D and H trees adopted different hydraulic strategies under drought stress. These different strategies might also imply different levels of resistance to xylem embolism in different individuals (Corcuera et al. 2011). Cavin et al. (2013) demonstrated substantial intraspecific variation in the response and recovery of *F. sylvatica* after an extreme drought event, suggesting that population-level intraspecific differences in drought response might be related to genotypic differences. The different behaviours of H and D trees could derive from different rooting depth or micro-environmental parameters experienced by different individuals. In fact, the Karst area has poorly developed top soils, but fissures and caves (often filled with clay) are very common, and their spatial distribution is not homogenous. Hence, it is possible that H and D trees had access to different water sources, as already shown for different woody angiosperms growing in the same area (Nardini et al. 2016). Another possible explanation for the observed differences might be related to different areas of origin of different individuals. Starting from the end of the 19th century, millions of trees have been planted in the Karst during afforestation activities. The hypothesis that the two groups of pines studied derive from different populations characterized by different genotypes cannot be excluded on the basis of our data.

The increased frequency of extreme drought events observed in recent decades has been the most likely factor leading to recent and widespread crown desiccation in *P. nigra* plantations in the Classical Karst. These events might lead to chronic stress and ultimately to severe decline of primary productivity and eventually tree death, as already observed for some trees. The analysis of the growth history of H and D trees, by means of dendrochronological techniques, wood anatomy and stable isotope analyses,

suggests the occurrence of two different hydraulic strategies adopted by the two groups of trees, i.e., a more conservative water use by H trees than by D trees. This diversity might be linked to different origins of the seedlings, but this hypothesis needs further testing. Also other possible factors, like different rooting depth, need further investigation. Indeed, our results are a promising starting point for future studies aimed at elucidating the physiological differences between *P. nigra* individuals that survived and those that succumbed to recent extreme droughts. The pinewoods in the Karst might represent a very interesting case of an unintentional long-term experiment, nowadays providing information about the possibility of selecting drought-resistant genotypes of forest species for a future warmer and drier climate.

Supplementary Data

Supplementary Data for this article are available at [Tree Physiology Online](http://www.treephysiology.org).

Acknowledgments

This study was funded by the University of Trieste (Finanziamento di Ateneo per la Ricerca Scientifica 2013 – Climate change and forest mortality: from physiological mechanisms to ecological consequences). L.P. was further supported by an Erasmus Placement grant. G.v.A. was supported by grants from the Swiss State Secretariat for Education, Research and Innovation SERI (SBFI C12.0100 and C14.0104). We are very grateful to Gai Petit and Tommaso Anfodillo (University of Padova) for invaluable help during field sampling of wood cores. We are grateful to Regione Friuli Venezia Giulia for authorization to collect wood sample from Bosco Bazzoni pines. This study was the result of A.N. and P.C. meeting together at a conference organized in Naples in April 2013 in the framework of the COST Action FP 1106 STReESS.

Authors' contribution

A.N., P.C. and L.P. planned and designed the research. L.P., P.C. and A.N. conducted fieldwork and performed dendrochronological experiments. L.P. and G.v.A. performed anatomical measurements. L.P. and M.S. performed isotopic analysis. All authors contributed to data elaboration and analysis. L.P. and P.C. wrote the manuscript, which was revised and finalized by A.N., with contributions by all authors.

Conflict of interest

None declared.

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