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## Research paper

# Effects of prescribed burning on ecophysiological, anatomical and stem hydraulic properties in *Pinus pinea* L.

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Prescribed burning (PB) is a widespread management technique for wildfire hazard abatement. Understanding PB effects on tree ecophysiology is key to defining burn prescriptions aimed at reducing fire hazard in Mediterranean pine plantations, such as *Pinus pinea* L. stands. We assessed physiological responses of adult *P. pinea* trees to PB using a combination of dendroecological, anatomical, hydraulic and isotopic analyses. Tree-ring widths, xylem cell wall thickness, lumen area, hydraulic diameter and tree-ring  $\delta^{13}\text{C}$  and  $\delta^{18}\text{O}$  were measured in trees on burned and control sites. Vulnerability curves were elaborated to assess tree hydraulic efficiency or safety. Despite the relatively intense thermal treatment (the residence time of temperatures above 50 °C at the stem surface ranged between 242 and 2239 s), burned trees did not suffer mechanical damage to stems, nor significant reduction in radial growth. Moreover, the PB did not affect xylem structure and tree hydraulics. No variations in  $^{13}\text{C}$ -derived water use efficiency were recorded. This confirmed the high resistance of *P. pinea* to surface fire at the stem base. However, burned trees showed consistently lower  $\delta^{18}\text{O}$  values in the PB year, as a likely consequence of reduced competition for water and nutrients due to the understory burning, which increased both photosynthetic activity and stomatal conductance. Our multi-approach analysis offers new perspectives on post-fire survival strategies of *P. pinea* in an environment where fires are predicted to increase in frequency and severity during the 21st century.

**Keywords:** carbon isotope, cavitation, intrinsic water use efficiency, oxygen isotope, radial growth, stone pine, wood anatomy, xylem hydraulic efficiency.

## Introduction

Prescribed burning (PB) is one of the most debated land management techniques, particularly in relation to the qualitative benefits of PB for wildfire hazard abatement, increased likelihood of asset protection and ecological impact on forest stands (North et al. 2015). Although a number of studies have assessed PB effects on different forest ecosystem components (e.g., McIver et al. 2012, Fernandes et al. 2013), several ecological concerns

remain (Fulé et al. 2004, Ryan et al. 2013). Prescribed burning has important impacts on forest soil and nutrients (McIver et al. 2012), forest structure (Moreira et al. 2003, Fernandes et al. 2008), seed banks (Keyser et al. 2012), understory vegetation (Arévalo et al. 2014) and overstorey trees mainly in terms of productivity (Hatten et al. 2012, Battipaglia et al. 2014b, Valor et al. 2015). However, little is known about the possible impacts of PB on water transport, tree water use efficiency (WUE), xylem hydraulic efficiency/safety and overall tree vulnerability to drought.

Heat stress caused by fire can potentially affect stem hydraulics via deformation of xylem tissue and/or by inducing embolism in xylem conduits (Michaletz et al. 2012). High temperature plumes during the fire (Kavanagh et al. 2010) can significantly increase xylem water tension, thus leading to 'air seeding' and embolism possibly causing strong reductions of root-to-leaf water transport capacity (Tyree and Zimmermann 2002, Lens et al. 2013). When embolism-induced loss of hydraulic conductivity exceeds a critical threshold, trees can undergo crown desiccation and even death (Tyree and Dixon 1986, Tyree and Sperry 1988, Savi et al. 2015). Moreover, heat and drought stresses coupled to xylem embolism can lead to prolonged stomatal closure and reduced photosynthetic rates (Tyree and Sperry 1988). Eventual heat-induced deformations of xylem conduit walls may also cause an increase in the xylem vulnerability to embolism formation (Michaletz et al. 2012), thus increasing the vulnerability of surviving trees to successive drought events (Savi et al. 2015). All these physiological effects have potentially important long-term consequences for surviving trees, since droughts are projected to increase in frequency, duration and severity with potential negative outcomes for many forest areas (Allen et al. 2010, Choat et al. 2012), in particular in the Mediterranean region, which has been identified as a climate change hotspot (Hulme et al. 1999, Giorgi and Lionello 2008).

Dendroecology has been widely used in the past to assess the effect of PB on trees' growth rates at multiple temporal scales (Peterson et al. 1994). Anatomical analysis of tree rings has been used to assess how environmental changes (such as temperature or water availability) influence xylem structure (Liang et al. 2013, Castagneri et al. 2015). In addition, xylem anatomy has been demonstrated to be good indicator of wood responses to severe fire events (De Micco et al. 2013, Battipaglia et al. 2014a). However, to the best of our knowledge, there are no studies on how PB influences the structure/function of xylem. Despite the fact that PB treatments are usually less intense than uncontrolled wildfires, the lethal temperature for cambial necrosis, above 60 °C at the stem level (Ventura et al. 1994), can be reached during PB. Furthermore, environmental impacts of fire on the soil microbiological and physical properties (Dumontet et al. 1996, Certini 2005) could negatively affect physiological processes related to xylogenesis. On the other side, the reduction of the shrub and herb biomass could enhance water availability to trees, possibly favoring the formation of an increasing number of xylem cells, with large lumen area, and thick wall (Martin-Benito et al. 2013), thus increasing xylem resistance to embolism, water transport and structural carbon storage capacity (Hacke et al. 2001, Fonti et al. 2010).

Finally, the analysis of the inter-relationships among  $\delta^{13}\text{C}$  and  $\delta^{18}\text{O}$  in tree rings has the potential to provide valuable functional and environmental information (Scheidegger et al. 2000, Barbour 2007, Battipaglia et al. 2007, 2010). In particular, in C3 plants,  $\delta^{13}\text{C}$  is a good proxy of leaf-level intrinsic WUE

( $\text{WUE}_i$ ), which is given by the ratio between net photosynthetic rate ( $A$ ) and stomatal conductance ( $g_s$ ) (Farquhar et al. 1982). Plant  $\delta^{18}\text{O}$  is influenced by source water  $\delta^{18}\text{O}$ , and is also inversely related to the ratio of atmospheric to leaf intercellular water vapor pressure ( $e_a/e_i$ ), and can thus provide a time-integrated indication of leaf  $g_s$  during the growing season (Barbour 2007). The combined analysis of plant  $\delta^{13}\text{C}$  and  $\delta^{18}\text{O}$  can provide valuable complementary information, as  $\delta^{18}\text{O}$  is related to  $g_s$ , but it is unaffected by  $A$ , and can thus help in separating the independent effects of  $A$  and  $g_s$  on  $\delta^{13}\text{C}$  and  $\text{WUE}_i$  (Scheidegger et al. 2000, Roden and Farquhar 2012, Battipaglia et al. 2013). Even if the interpretation of the double model  $\delta^{13}\text{C} - \delta^{18}\text{O}$  is not straightforward (Gessler et al. 2014), it can still be informative when applied in strongly water-limited ecosystems such as the Mediterranean biome (Moreno-Gutiérrez et al. 2012, Gessler et al. 2014, Altieri et al. 2015).

The present study aims to evaluate the short-term physiological effects (a few weeks to a few months) of relatively intense PB on *Pinus pinea* L. (stone pine), a widespread and fire-prone species, largely introduced for plantations in the Mediterranean Basin area (Mazza and Manetti 2013), where understory burning is a common practice for fire hazard reduction (Ascoli and Bovio 2013, Fernandes et al. 2013). Previous studies pointed to higher resistance to surface fire of *P. pinea* in relation to the other Mediterranean pines (Pausas et al. 2004, Rigolot 2004, Fernandes et al. 2008), and to a low rate of mortality among adult individuals following fire events. Thus, we hypothesize that burned trees would not be damaged at the stem and cambium level, and radial growth would not be severely affected by the experimental fire. Further, PB could reduce competition for water by removing burning coexisting shrubs and herbaceous species. This could increase WUE and water transport, provided heat stress does not reduce hydraulic efficiency or safety as previously hypothesized by Michaletz et al. (2012). A detailed assessment of the level of heat stress suffered by stems during PB and a combination of ring width, wood anatomical, hydraulic and stable isotopes analyses have the potential to contribute to a thorough understanding of possible impacts of this forest management technique on tree health status.

## Materials and methods

### Study area

The study was carried out in a *P. pinea* plantation at Castel Volturno Natural Reserve (40°57'N; 13°59'E; 8 m above sea level), one of the most fire-prone areas in Southern Italy ([www://burc.regione.campania.it](http://www.burc.regione.campania.it)). The climate of the area is typically Mediterranean with hot and dry summers. Monthly mean temperature and precipitation for the period 1974–2012 are available from the meteorological station of Ischitella, located 200 m from the sampling site. The mean annual temperature is 13.6 °C, with mean winter and summer temperatures of 6.7 and 21.0 °C,

respectively. Total rainfall averages 761.3 mm per year, mostly falling in autumn and winter. Between 2005 and 2014, the years 2007 and 2012 were characterized by high temperature and low precipitation (Figure 1).

*Pinus pinea* plantations are mostly located on the southern part of the Reserve, with a canopy cover of ~80%. The trees were planted in the early 1960s, when a large-scale afforestation was carried out along the Italian coasts for the consolidation of sand dunes and to protect soil and crops from erosion by salty winds. Nowadays these plantations are an important cultural landscape, included in the EU priority habitat 2270 for its considerable size reduction along all the Mediterranean coast, and are maintained by the Natural Reserve mainly for recreational purposes. Consequently, interventions for wildfire hazard abatement are planned to reduce the fire risk during the summer when tourism increases in the area.

The understory consists of flammable shrubs such as *Phyllirea angustifolia* L., *Rhamnus alaternus* L. and *Asparagus acutifolius* L., and a very thick needle litter layer (~6–10 cm).

### Prescribed burning experiment

The experimental design was based on two sites ~0.5 ha each. The first site (BS) was subjected to a relatively intense PB, while a second control site (CS) was separated by BS by a buffer area 25 m wide. To exclude other environmental influences on the stands apart from the PB, care was taken to select sites with the same tree density (400 trees ha<sup>-1</sup>), aspect, elevation and soil features. At both BS and CS sites, we selected 20 trees to carry out the analyses described hereafter (diameter = 40 ± 11 cm; mean height = 14 ± 1 m; mean bark thickness at the base of the trunk = 3.1 ± 0.75 cm; all values are mean ± SD). At BS, litter yield on a dry mass basis, as assessed by destructive sampling (Catchpole and Wheeler 1992) in five experimental units (1 m<sup>2</sup>), was 6.4 t ha<sup>-1</sup>. In order to increase the burn severity, 2 kg of dry litter was added onto an area of ~4 m<sup>2</sup> (i.e., +5 t ha<sup>-1</sup>) around the base of the trunk of each tree, to increase the fuels available for the combustion and thus peak tempera-

tures and residence time along the stem. Each of the selected 20 trees was equipped with two K-type thermocouples (temperature range: 0–1200 °C; accuracy: ±4.0 °C; diameter: 0.4 mm) at the stem base (30–50 cm above the ground, with an angle of 120° between them). At this height, the heat stress due to a surface fire was expected to be the most intense, with highest temperatures and maximum duration (Fahnestock and Hare 1964). Thermocouples were fixed to adhere to the bark surface within one of the trunk fissure at the stem base (Figure 2). Each thermocouple was connected to a datalogger (Hobo-Onset, Massachusetts, USA) buried at the tree base, recording temperature at a frequency of 1 s.

The PB experiment was carried out on 12 March 2014. Burning lasted from 10:30 to 17:00 h. On-site weather data were measured every 30 min with a portable weather station. Average environmental conditions during the burn were: air temperature: 18 °C; relative humidity: 54%; wind speed: 1–3 km h<sup>-1</sup>; and days since last rain: 4. Due to safety protocols in the Nature Reserve, the ignition technique was based on a backfire, i.e., against the wind. Consequently, flame length at the base of the trunk never exceeded 1.5 m despite litter fuel addition. Still, flame length was at the upper limit recommended for PB in Mediterranean pine plantations (Ascoli and Bovio 2013, Fernandes et al. 2013).

### Tree-ring sampling

In December 2014 (i.e., 9 months after the PB), the selected 20 *P. pinea* were sampled from each site. Three cores per tree were extracted along the trunk (30–50 cm above the ground with an angle of 120° between them) with a 5 mm increment borer (Haglöfs, Langsele, Sweden); two cores were used for dendroisotopic measurements and the third one for anatomical analysis.

Cores were polished using sand paper of different grain-size, and ring-width (TRW) measurements were made at a resolution of 0.01 mm, using LINTAB measurement equipment fitted with a stereoscope and equipped with TSAP software (Frank Rinn,

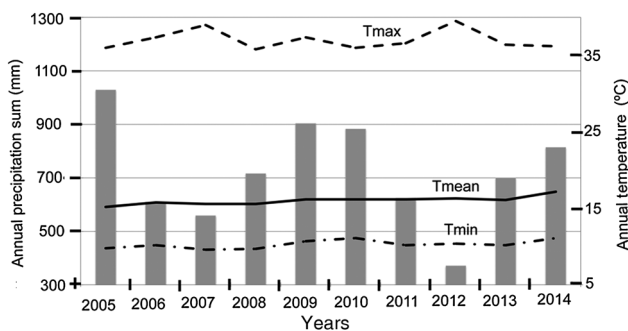


Figure 1. Maximum, mean and minimum temperature and precipitation sums from 2005 to 2014 recorded at the meteorological station of the Ischitella site. Solid and dotted lines represent temperatures (°C) and bars represent precipitation (mm).



Figure 2. Tree equipped with thermocouples fixed at stem base. Note the complete fuel consumption at the stem base after the PB.



Heidelberg, Germany). After visual cross-dating to identify common marker years and ring-width patterns (Schweingruber 1996), samples from each site were correlated according to Gleichläufigkeit, a statistical measure of the year-to-year agreement between the interval trends of the chronologies based on the signing of the agreement (Kaennel and Schweingruber 1995) and a Student's *t*-test to assess the degree of correlation between curves. The program COFECHA (Holmes 1983) was run to validate the cross-dating and measurements, as well as to highlight potential errors. Once all measurement series had been validated, tree-ring chronologies were developed. Series were detrended with a 10-year spline to remove long-term growth trends embedded in the raw tree-ring series that were thought to be induced by non-climatic influences, such as aging and competition between trees (Fritts 1976). Tree-ring indices were calculated as residuals from the estimated age trend. The signal-to-noise ratio, which is an expression of the strength of the observed common signal among the trees in the chronology, the expressed population signal (EPS), which indicates the level of coherence of the constructed chronology and how it portrays the hypothetical perfect population chronology, and the mean interseries correlation, which is a measure of the common variance between the single series in a chronology, were calculated for the *P. pinea* chronologies of both sites using 30-year common interval with an overlap of 15 years (Table 1). Furthermore, the mean sensitivity, which is a measure of the year-to-year variability in the master chronology, and the series intercorrelation, which is a measure of the stand level signal, were evaluated (Table 1).

### Anatomical parameters

For the analysis of xylem anatomical parameters, a subsample of 10 cores, corresponding to five trees at BS and five trees at CS, were selected among those (20 for each site) without evident irregularities such as nodes. Anatomical analyses were performed for rings produced in the period 2005–14, in order to include data before treatment and following the PB. The cores were divided into 4- to 5-cm-long pieces. At the TeSAF Department (University of Padua) laboratory, transversal microsections (12–14  $\mu\text{m}$  thick) were obtained with a rotary microtome (Leica, Heidelberg, Germany), stained with safranin (1% in distilled water) and fixed on permanent slides with Eukitt (BioOptica,

Table 1. Dendrochronological characteristics of the ring-width chronologies of *P. pinea* at BS (burned site) and CS (control site). EPS, expressed population signal; SNR, signal-to-noise ratio; RBAR, mean interseries correlation; S.I., series intercorrelation; MS, mean sensitivity.

	CS	BS
EPS	0.93	0.98
SNR	17	16
RBAR	0.85	0.86
S.I.	0.7	0.68
MS	0.21	0.15

Milan, Italy). Digital images were captured with a light microscope at  $\times 100$  magnification (Nikon Eclipse 80 mounted with distortion-free oculars), and stitched together with PTGui software (New House Internet Service B.V., Rotterdam, The Netherlands). The images, with a resolution of 2.074 pixels  $\mu\text{m}^{-1}$ , were then processed with the image analysis software ROXAS (version 2.0; von Arx and Carrer 2014) to automatically measure the lumen size and the wall thickness (as the mean of four perpendicular radii) of all the cells in the image. In total, >150,000 cells were measured.

For each dated annual ring, we assessed: (i) the mean cell wall thickness (CWT,  $\mu\text{m}$ ); (ii) the mean cell lumen area (CA,  $\mu\text{m}^2$ ); and (iii) the mean hydraulic diameter (Dh,  $\mu\text{m}$ ), according to the equation:

$$Dh = \frac{\sum_{n=1}^N D_n^5}{\sum_{n=1}^N D_n^4}$$

where  $D_n$  is the lumen diameter of the cell  $n$ . Dh is commonly used to assess potential xylem water transport capacity, as it is proportional to hydraulic conductivity defined according to the Hagen–Poiseuille equation as the fourth power of the pore radius (Sperry et al. 1994, Tyree and Zimmermann 2002). For each ring, mean and standard deviation of CWT, CA and Dh of trees at BS and CS were calculated separately.

For a detailed anatomical analysis of the wood formed after PB, we assessed CWT and CA within 20 tangential sectors of equal radial width for the 2014 rings. For CS and BS separately, the average CA (and CWT) value of each sector in the five trees was used to build the mean ring profile of CA (and CWT).

### Hydraulic measurements

One month after the PB, tree sampling for hydraulic analyses was carried out at both BS and CS. Five trees per site were selected and stem sections ( $\sim 25$  cm in length) were cut using a chainsaw at a height of 30–50 cm above the ground. The samples were wrapped in wet filter paper and cling film in order to avoid dehydration and immediately shipped to the Institute of Botany, University of Innsbruck (Austria).

Trunk samples were split using a chisel and a hammer. Approximately 25 cm long and 2 cm wide longitudinal sticks of the most outer wood portion (3-/5-year-old rings) were prepared for hydraulic measurements. Only wood including 3-/5-year-old rings was selected, because it represents the most exposed portion to the heat stress during the fire. Sample ends were recut under water several times using shears and a wood carving knife to the final length of  $\sim 15$  cm, and inserted into a custom-built rotor (designed by J.S. Sperry) (Li et al. 2008), of a Sorvall RC-5 centrifuge (Thermo Fisher Scientific, Waltham, MA, USA). Vulnerability curves were then elaborated using the Cavitron technique (Cochard et al. 2005). Pit aspiration was

avoided by following the protocol of Beikircher et al. (2010). The relationship between water potential ( $\Psi$ , MPa) and the percentage loss of hydraulic conductivity (PLC, %) was used to extrapolate the reference values  $P_{12}$ ,  $P_{50}$  and  $P_{88}$ , i.e., the xylem water potential inducing 12, 50 and 88% PLC, respectively.

### Carbon and oxygen isotopes

From each sampled core, we selected the section corresponding to the period 2005–14, in order to include data before treatment and after the PB. Samples were annually divided and ground with a centrifugal mill (ZM 1000, Retsch, Haan, Germany) using a mesh size of 0.5 mm to assure homogeneity. Cellulose was extracted with a double-step digestion (Boettger et al. 2007, Battipaglia et al. 2008).

The carbon and oxygen stable isotope composition was measured at the CIRCE Lab (Center for Isotopic Research on the Cultural and Environmental Heritage, Caserta, Italy) by continuous-flow isotope ratio mass spectrometry (Delta V plus, Thermo Electron Corporation, Bremen, Germany) using 0.06 mg of dry matter for  $^{13}\text{C}$  measurements and 0.3 mg for  $^{18}\text{O}$  determinations. The  $\delta^{13}\text{C}$  series were corrected for the Suess Effect (decrease in  $\delta^{13}\text{C}$  of atmospheric  $\text{CO}_2$  since the beginning of industrialization) resulting from the emission of fossil carbon dioxide, which is depleted in  $^{13}\text{C}$  (Francey et al. 1999, McCarroll and Loader 2004). The corrected series were used for all statistical analyses.

Isotopic compositions are expressed in delta notation (‰) relative to accepted reference standards: Vienna PeeDee Belemnite for carbon isotope values and Vienna Standard Mean Ocean Water for oxygen isotope values. The standard deviation for the repeated analysis of an internal standard (commercial cellulose) was lower than 0.1‰ for carbon and lower than 0.3‰ for oxygen.

### Intrinsic WUE and oxygen isotope

The analysis of carbon stable isotopes in tree rings is a powerful tool for the estimation of annual variability of  $\text{WUE}_i$ , because  $\delta^{13}\text{C}$  values reflect the  $c_i/c_a$  ratio, where  $c_i$  is the partial pressure of  $\text{CO}_2$  in leaf intracellular space and  $c_a$  is the partial pressure of  $\text{CO}_2$  in the atmosphere (Farquhar et al. 1982). Limited water availability or other factors reduce  $g_s$  and photosynthetic rate ( $A$ ), thus inducing changes in  $\text{WUE}_i$  and in  $c_i$  that are recorded by variations in  $\delta^{13}\text{C}$  in assimilated  $\text{CO}_2$  and plant tissue (Seibt et al. 2008, Maseyk et al. 2011). The  $\delta^{18}\text{O}$  of plant tissue can be influenced by differences in  $\delta^{18}\text{O}$  of source water, variation in  $\delta^{18}\text{O}$  of water vapor in the air, evaporative enrichment at the evaporation sites within the leaf and mixing of  $\delta^{18}\text{O}$  from evaporated and unevaporated source water during synthesis of carbohydrates (Barbour et al. 2000, Roden and Ehleringer 2000, Treydte et al. 2014).

The evaporative enrichment of leaf water is strongly affected by changes in  $g_s$  (Barbour 2007) and produces a strong signal that is reflected in the  $\delta^{18}\text{O}$  values of tree rings. Measuring plant  $\delta^{18}\text{O}$  can thus help to separate the independent effects of  $A$  and  $g_s$  on  $\delta^{13}\text{C}$  (Scheidegger et al. 2000).

In this article,  $\delta^{13}\text{C}$  in tree rings was used to calculate the  $\text{WUE}_i$ , which is defined as the ratio between net photosynthesis ( $A$ ) and  $g_s$  (Ehleringer et al. 1993)

$$\text{WUE}_i = \frac{A}{g_s} = \frac{c_a - c_i}{1.6} \quad (1)$$

with  $c_a$  and  $c_i$  being the  $\text{CO}_2$  concentrations in the air and in intercellular spaces, respectively, and 1.6 being the ratio of diffusivity of water and  $\text{CO}_2$  in air.

For C3 plants,  $c_i$  can be estimated from  $\delta^{13}\text{C}$  in plant organic matter ( $\delta^{13}\text{C}_{\text{sample}}$ ), which is related to  $\delta^{13}\text{C}$  of atmospheric  $\text{CO}_2$  ( $\delta^{13}\text{C}_a$ ) and the ratio of  $c_i/c_a$  (Farquhar et al. 1989):

$$\delta^{13}\text{C}_{\text{sample}} = \delta^{13}\text{C}_a - a - \frac{(b-a)c_i}{c_a} \quad (2)$$

where  $a$  is the fractionation for  $^{13}\text{CO}_2$  resulting from diffusion through air (4.4‰) and  $b$  is the fractionation during carboxylation (27‰). Thus,  $c_i$  can be derived as follows:

$$c_i = c_a \frac{\delta^{13}\text{C}_a - \delta^{13}\text{C}_{\text{sample}} - a}{b - a} \quad (3)$$

We used measured  $\delta^{13}\text{C}_{\text{air}}$  values for the period 2004–14 available online (<http://www.esrl.noaa.gov/gmd/>);  $c_a$  is the concentration of  $\text{CO}_2$  in the atmosphere, estimated for each year and obtained by NOAA (<http://www.esrl.noaa.gov/>, Mauna Loa station).

Finally, replacing  $c_a$  and  $\delta^{13}\text{C}_a$  in Eq. (3) allowed us to estimate  $c_i$ , and Eq. (1) may be solved as:

$$\text{WUE} = \frac{c_a}{1.6} \frac{b - \delta^{13}\text{C}_a + \delta^{13}\text{C}_{\text{sample}}}{b - a} \quad (4)$$

### Statistical analyses

To characterize the heating treatment during the PB experiment, thermocouple data were used to calculate the following variables: (i) maximum temperature reached at the bark surface; (ii) time–temperature integration value ( $\text{s}^\circ\text{C}$ ) for each tree, when the temperature at the bark surface exceeded 50 °C; and (iii) duration of high temperature at the bark surface above different thresholds commonly used in literature (50, 100 and 300 °C) (e.g., Castagneri et al. 2013). The temperature residence time above 300 °C (indicative of flaming combustion; Wotton et al. 2011) was compared with the time needed to kill the cambium ( $T_c$ ) when a constant temperature is applied at the bark surface (Dickinson and Johnson 2001), i.e., the time needed until the heat transfers through the bark and raises the internal temperature of the cambium up to 60 °C (point of necrosis). We computed  $T_c$  (in seconds) by commonly used models for one-dimensional heat transfer in the bole as a function of bark

thickness: (i)  $2.9 \times 60 \times x^2$ , where  $x$  is the bark thickness in cm, i.e., Equation 10 in Peterson and Ryan (1986); and (ii)  $1.7 \times 10^6 \times (x/100)^2$ , where  $x$  is the bark thickness in cm, i.e., after resolving Equation 13 in Dickinson and Johnson (2001), imputing an ambient temperature of 18 °C, a fire temperature at the bark surface of 300 °C, a temperature of 60 °C at which vascular cambium necrosis would occur and a thermal diffusivity of the bark at  $1.35 \times 10^{-7} \text{ m}^2 \text{ s}^{-1}$  (Dickinson and Johnson 2001).

To identify the effects of treatment (burned vs control), climate (mean annual temperature and precipitation sum) and their interaction on TRW, WUE<sub>i</sub>,  $\delta^{18}\text{O}$ , CA, CWT and Dh (dependent variables), a mixed model analysis of variance was used, to account for the random sources of variation associated with repeated measures. The analysis was applied using the package XLSTAT 2010 (Microsoft Corp., Seattle, WA, USA).

## Results

### Heating treatment

Temperatures recorded at the bark surface 30–50 cm above ground allowed assessment of the magnitude of heating treatment during the PB experiment. A broad gradient was attained: the time–temperature integration above 50 °C ranged between 27,825 and 595,948 s °C. The maximum temperature range was 361–851 °C, but peaks were reached only for few seconds. The residence time of temperatures above 50 and 300 °C ranged between 242–2239 and 0–1323 s (Figure 3), respectively, and on average was 826 and 165 s, respectively. Fuel consumption was complete at the base of most stems (Figure 2). During the combustion, we observed flaming bark plaques twisting, becoming unhooked from the trunk and dropping to the ground at most trees (see Video available as Supple-

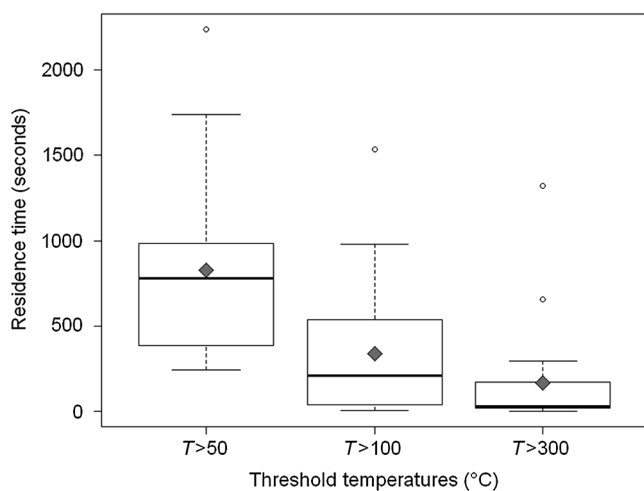


Figure 3. Box plot of temperature distribution at the bark surface of trees during the PB experiment. Gray points indicate mean values. Whiskers extend to the most extreme data point, which is 1.5 times the range of the interquartile from the box. Open circles are values that exceed whiskers.

mentary Data at *Tree Physiology* Online), indicative of the high fire severity at the base of the trunk. Still, no individuals sampled at the BS presented any fire scars.

Considering that selected trees had an average bark thickness of 3.1 cm, the time needed to cause the necrosis of the vascular cambium according to Peterson and Ryan (1986) and Dickinson and Johnson (2001) models was calculated to be 1672 and 1634 s, respectively, for an exposure at a constant temperature of 300 °C.

### Prescribed burning effect on tree growth

Trees at BS and CS belonged to the same age class, with a mean of  $50 \pm 7$  years. High EPS (Table 1) values ( $>0.85$ ) in all age classes and sites indicated that the chronologies were representative of radial growth variations of the whole population of trees (Wigley et al. 1984). All the statistics listed in Table 1 showed the strong common growth signal between the two sites and high year-to-year radial growth variability associated with interannual changes in climatic conditions.

The TRW chronologies of BS and CS showed a similar trend (Figure 4), and their variations correlated significantly ( $r = 0.77$ ,  $P < 0.01$ ) during the whole growing period and in particular during the period 2005–13 ( $r = 0.97$ ,  $P < 0.001$ ).

### Anatomical parameters

Mean CA, Dh and CWT were not different between CS and BS in any year (2005–13) before the fire event (Figure 5). Moreover, their interannual variations were synchronized between CS and BS

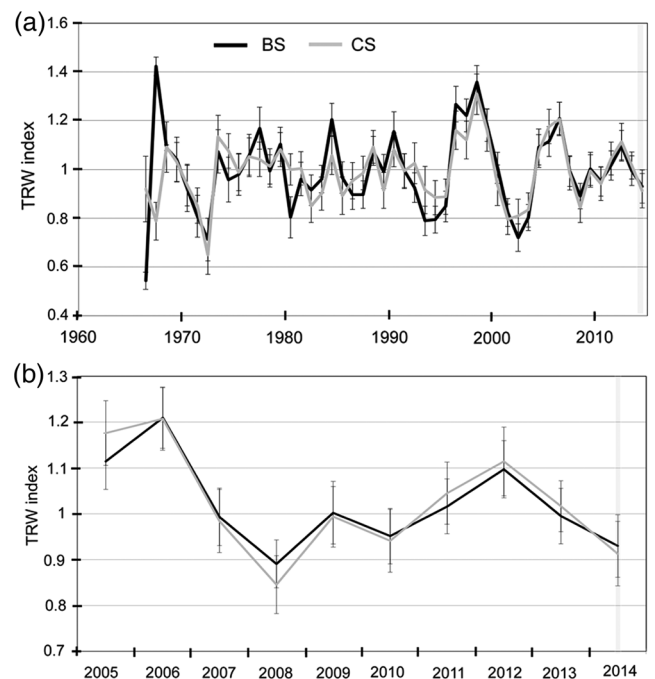


Figure 4. Mean TRW index with SD of *P. pinea* individuals at the burned site (BS—black line) and the control site (CS—gray line) during (a) the whole life period and (b) during the 2005–14 period. The prescribed fire year is indicated by gray shading (2014).

( $r = 0.88$ ,  $P < 0.001$ , for CA;  $r = 0.63$ ,  $P < 0.05$ , for Dh;  $r = 0.69$ ,  $P < 0.05$ , for CWT), indicating that xylem structure in the two sites was similarly affected by environmental variations. Fire did not cause any mechanical damage to stems, as broken or irregular shaped cells, or resin ducts, were not observed in the 2013 (previous year) and 2014 (successive year) rings at BS (see Figure S1 available as Supplementary Data at *Tree Physiology* Online). After the PB (ring 2014), CA and Dh were not statistically different ( $P > 0.05$ ) between CS (CA = 584  $\mu\text{m}^2$ ; Dh = 39.2  $\mu\text{m}$ ) and BS (CA = 630  $\mu\text{m}^2$ ; Dh = 40.6  $\mu\text{m}$ ) (Figure 5a and b). Similarly, the analysis of the ring profiles did not reveal significant differences between the two sites (Figure 6a). For CWT (Figure 5c), similarity between the plots was even more apparent (CWT = 5.38  $\mu\text{m}$  at CS; CWT = 5.40  $\mu\text{m}$  at BS for the 2014 ring). The mean CWT profiles were similar too, and moderate differences in the last ring sectors were not significant (Figure 6b).

**Hydraulic parameters**

The reference values  $P_{12}$ ,  $P_{50}$  and  $P_{88}$  extrapolated from vulnerability curves were found to be  $-2.40 \pm 0.38$ ,  $-3.29 \pm 0.12$  and  $-3.83 \pm 0.23$  MPa, and  $-2.30 \pm 0.48$ ,  $-3.17 \pm 0.30$  and  $-3.87 \pm 0.37$  MPa for control (CS) and burned trees (BS), respectively. No statistically significant differences ( $P > 0.05$ ) were observed in terms of  $P_{12}$ ,  $P_{50}$  and  $P_{88}$ , or the vulnerability

curve slope ( $P_{88}-P_{12}$ ) between experimental groups (Figure 7). The value of  $P_{50}$  of *P. pinea* averaged  $-3.22 \pm 0.23$  MPa, while maximum hydraulic specific conductivity ( $K_s$ ) was similar in CS and BS sites with an average of  $5.1 \pm 1.6 \times 10^{-3} \text{ m}^2 \text{ s}^{-1} \text{ MPa}^{-1}$ .

**Intrinsic water use efficiency and oxygen isotopes**

During 2014, the PB influenced  $\delta^{18}\text{O}$  at BS, while the interaction between climate and treatment had a significant effect not only on  $\delta^{18}\text{O}$  but also on  $\text{WUE}_i$ .  $^{13}\text{C}$ -derived  $\text{WUE}_i$  did not differ between control and burned trees either in the period 2005–13 or in the post-fire year (Figure 8a). The  $\delta^{18}\text{O}$  analysis revealed common values during 2005–13 and a significant decrease at the BS following the PB (Figure 8b).

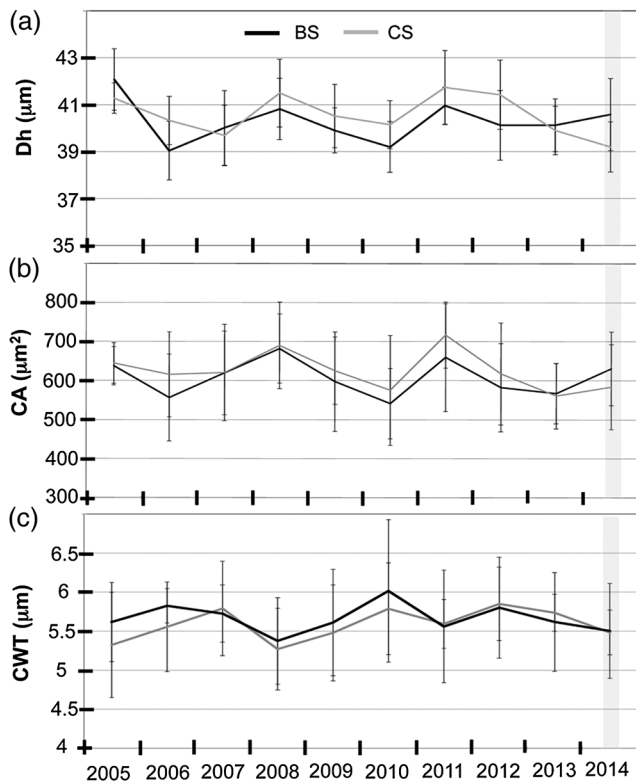


Figure 5. Mean values and SD of (a) Dh, (b) CA and (c) CWT assessed from five *P. pinea* trees at the burned site (BS—black line) and the control site (CS—gray line) during the 2005–14 period. Gray shading indicates the prescribed fire year (2014).

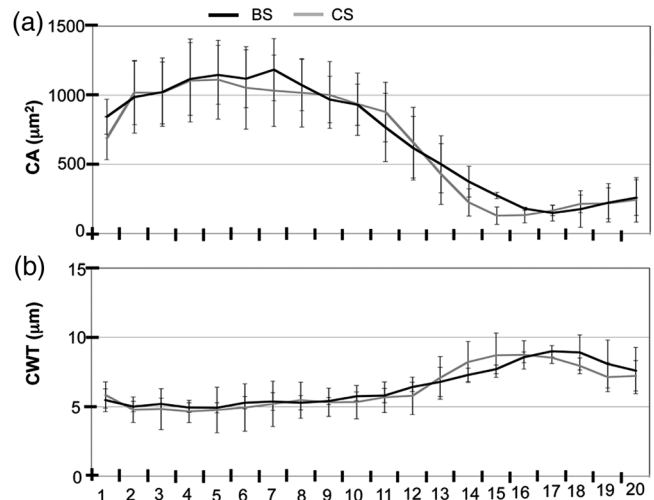


Figure 6. Cell lumen area (CA) and CWT mean (with SD) profiles for the year 2014 at the burned (BS) and the control (CS) site. For CS and BS separately, the average CA and CWT values of each ring sector in the five trees were used to build the mean ring profile of CA and CWT, respectively.

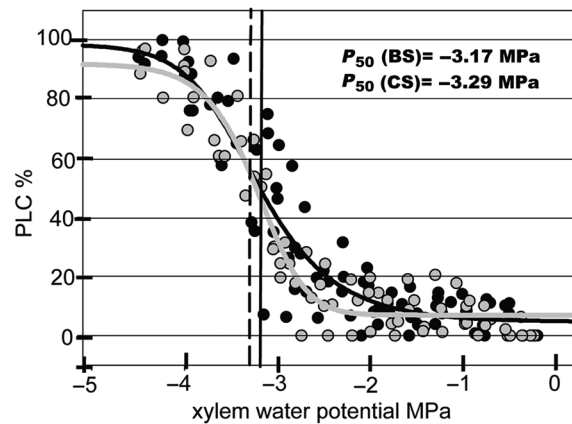


Figure 7. Vulnerability curves reporting the relationship between xylem water potential ( $\Psi$ ) and PLC of burned *P. pinea* trees (black circles, BS) and control trees (gray circles, CS). The sigmoidal regressions and the extrapolated  $\Psi$  values inducing 50% loss of hydraulic conductivity ( $P_{50}$ ) are reported (solid vertical line, BS; dashed vertical line, CS).



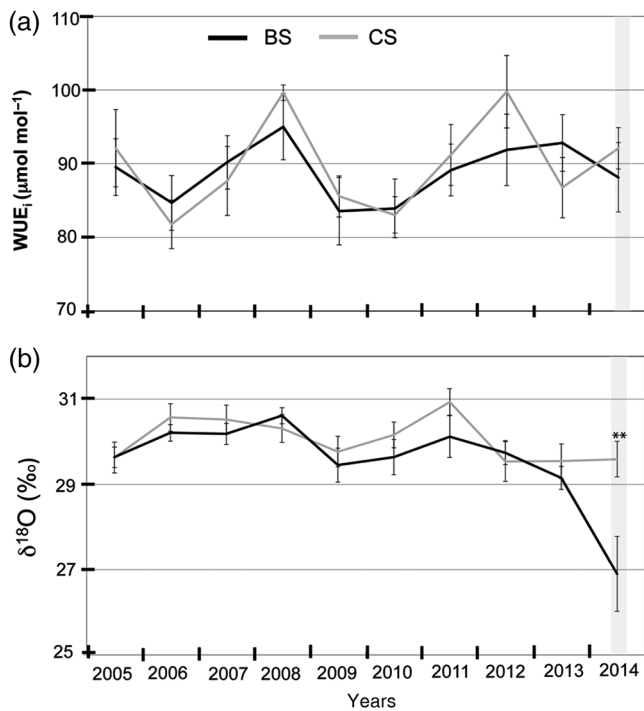


Figure 8. Values of (a)  $WUE_i \pm SD$  ( $\mu\text{mol mol}^{-1}$ ) and (b) cellulose  $\delta^{18}\text{O} \pm SD$  (‰) during the period 2005–14 of *P. pinea* individuals at the burned site (BS—black line) and the control site (CS—gray line). Gray shading indicates the prescribed fire year (2014). Significance between treatments (burned vs control) is indicated as \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

The  $WUE_i$  values of trees growing in BS and CS were closely related to the corresponding  $\delta^{18}\text{O}$  values ( $r = 0.68$ ,  $P < 0.05$ ) during the entire study period, including the PB year. A significant correlation was found between  $WUE_i$  and the corresponding TRW values at both sites ( $r = 0.62$  for CS,  $r = 0.63$  for BS,  $P < 0.05$ ).

Results from the mixed model analysis of variance (Table 2) during the 2005–14 period confirmed that most of the parameters (TRW, CA, CWT, Dh and  $WUE_i$ ) were not influenced by the PB treatment, while they were influenced by climatic parameters in 2007 and 2012.

## Discussion

### *Pinus pinea* resistance to PB

*Pinus pinea* trees were shown to be resistant to the PB treatment. By increasing dry litter at the base of trunks, we subjected plants to a heat treatment comparable to relatively intense burn experiments in pine plantations (Fahnestock and Hare 1964, Rego and Rigolot 1990, Butler and Dickinson 2010). For example, during a headfire in *Pinus palustris*, Fahnestock and Hare (1964) recorded at the bark surface, 30 cm above ground, a temperature residence time above 300 °C of 200 s, i.e., similar to values recorded in the present study.

Previous studies (Rodrigo et al. 2004, 2007, González et al. 2007) reported that *P. pinea* has a high fire resistance when

compared with other Mediterranean pines (Fernandes et al. 2008), particularly when the crown is not scorched (Rigolot 2004), as in the present study. Rigolot (2004), studying *P. pinea* mortality after a wildfire in southern France, pointed out that this species is more resistant to surface fires than the other pines, thanks to the thicker bark for a given stem diameter, and consequently the improved stem insulation from heat. Indeed, bark thickness is a determinant of the degree of cambium damage at any given heat treatment (Dickinson and Johnson 2001, van Mantgem and Schwartz 2003, Bova and Dickinson 2009). In our study, trees selected for the experiment had an average bark thickness of 3.1 cm, which would require a time of exposure at 300 °C of between 1672 and 1634 s to provoke vascular cambium necrosis, depending on the heating model considered (Peterson and Ryan 1986, Dickinson and Johnson 2001). We recorded a maximum time of exposure above 300 °C of 1323 s, but on average, it was 165 s. Considering that thermocouples placed at the bark surface overestimated the net heat flux (i.e., inward minus outward energy flow) through the bark (Bova and Dickinson 2009), the difference between the modeled required temperature and the real one could be even larger. In addition to the insulating effect, the highly creviced and rough bark of adult pines may have contributed to limited stem heating by dispersing the heat (Fahnestock and Hare 1964, Nicolai 1986, Bauer et al. 2010, Odhiambo et al. 2014). Concurrently, the flaming bark dropping that was observed in the present study (see Video available as Supplementary Data at *Tree Physiology* Online), may have also reduced the combustion duration at the bark surface, thus increasing the thermal insulation and resistance to fire injury.

### Effect of PB on xylem hydraulics

Prescribed burning did not cause any evident damage or wound on the tree trunks. The anatomical analysis of tree rings showed that xylem structure of trees at BS was not affected, as the rings formed before (2013) and after (2014) the fire did not show any altered or broken cells nor traumatic resin ducts (see De Micco et al. 2013 for an anatomical description of wood reactions to wildfires in *Pinus halepensis*).

However, heat stress can have less evident but long-lasting effects on tree functioning (Beghin et al. 2011, Battipaglia et al. 2014b). Our detailed analysis of xylem anatomy revealed that fire did not even influence the cell wall thickness of the 2014 ring. Therefore, during the first year after the PB, trees' ability to allocate structural carbon in the xylem cell walls was unaffected. Fire exposure did not influence an important component of xylem resistance to hydraulic failure, i.e., cell wall thickness of earlywood cells. Indeed, thick walls are required to withstand the negative pressures caused by water stress (Martin-Benito et al. 2013), especially in large vessels prone to embolism risk. Furthermore, fire did not alter the mean hydraulic diameter, indicative of the rings' potential hydraulic conductivity, nor the profile



Table 2. Effects of treatments (burned vs control), climate (temperature and precipitation) and their interaction on TRW, WUE,  $\delta^{18}\text{O}$ , Dh, CA and CWT from mixed model analysis of variance for the period 2005–14. TRW, WUE,  $\delta^{18}\text{O}$ , Dh, CA and CWT were included as response variables. df, degrees of freedom; n.s., not significant. Only the significant  $F$  values are reported with the corresponding  $P$  value, where \* corresponds to  $P < 0.05$ , \*\* corresponds to  $P < 0.01$  and \*\*\* corresponds to  $P < 0.001$ .

Years	Effect	df	TRW	WUE <sub>i</sub>	$\delta^{18}\text{O}$	Dh	CA	CWT
2005	Treatments	1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	Climate	1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	Interaction	1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
2006	Treatments	1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	Climate	1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	Interaction	1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
2007	Treatments	1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	Climate	1	43.671***	23.782***	37.673***	25.980***	34.141***	15.182**
	Interaction	1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
2008	Treatments	1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	Climate	1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	Interaction	1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
2009	Treatments	1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	Climate	1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	Interaction	1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
2010	Treatments	1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	Climate	1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	Interaction	1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
2011	Treatments	1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	Climate	1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	Interaction	1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
2012	Treatments	1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	Climate	1	51.782***	60.522***	32.651***	30.764***	40.231***	31.49***
	Interaction	1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
2013	Treatments	1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	Climate	1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	Interaction	1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
2014	Treatments	1	n.s.	n.s.	140.3***	n.s.	n.s.	n.s.
	Climate	1	n.s.	60.525	76.823***	n.s.	n.s.	n.s.
	Interaction	1	n.s.	82.65***	122.23***	n.s.	n.s.	n.s.

of cell lumen area in the 2014 ring. This not only indicated the absence of heat damage at the cambial level but also suggested that fire did not reduce water supply to trees in the stand. Indeed, the cell lumen area along the ring profile results from the cell expansion phase along the growing season, which is largely driven by water availability, especially in dry environments (Wilkinson et al. 2015).

Our data also suggest that heat stress (BS site) did not decrease the hydraulic efficiency/safety of the wood already formed before the treatment. The vulnerability threshold of  $P_{50}$  for *P. pinea* was found to be around  $-3.20$  MPa at both sites, in accordance with previous studies (Oliveras et al. 2003). The species in the genus *Pinus*, and *P. pinea* in particular, tend to be more vulnerable to xylem embolism than most other conifers (Martínez-Vilalta et al. 2004, Choat et al. 2012, Bouche et al. 2014). The lack of effects could be due to the relatively low fire severity in the present PB, which had a minor impact on the short-term growth and ecophysiology of pine forests. However, a high vulnerability to embolism, typical of this species, seems to have implications for whole-plant biomass allocation and for

the physiological strategies used to face water stress (Davis et al. 1998). A previous study by Michaletz et al. (2012) reported significant effects of heat on xylem hydraulic efficiency and vulnerability to embolism in *Populus balsamifera*. In particular, they observed that heating caused deformation of conduit walls leading to significant reductions in xylem conductivity and increased vulnerability to embolism (Michaletz et al. 2012). Our data, obtained on a conifer tree, do not support these conclusions, in that we did not observe significant anatomical or hydraulic differences between control (CS) and burned (BS) trees. Further studies are required to check whether the discrepancy arises because of different angiosperm vs gymnosperm responses to fire at the xylem level, or because of the treatment applied by Michaletz and coauthors in their study (i.e., 5-min-long heating at different temperatures followed by immediate immersion of stems in an ice bath). Of course, on the basis of our data, we cannot exclude longer term impacts on wood production after the fire event.

Our data confirmed the sensitivity to climate of *P. pinea* (Campelo et al. 2007, De Luis et al. 2009, Mazza and Manetti

2013). All analyzed parameters were influenced by dry years (2007, 2012), while the interaction between drought and fire had an effect on  $WUE_i$  and  $g_s$  of this species. Apparently, *P. pinea* can extract water from the soil very quickly when this resource is available, but it also needs mechanisms to limit water use under drought (Martínez-Vilalta et al. 2004). In previous studies, the relatively high vulnerability to embolism of *P. pinea* was suggested to be compensated by an efficient stomatal control of water use (Linton et al. 1998, Rundel and Yoder 1998), and Oliveras et al. (2003) hypothesized that the high hydraulic efficiency of *P. pinea* allows this species to have quite strict stomatal control.

### Effect of PB on $WUE_i$ and stomatal activity

We found that trees at the BS site showed consistently lower  $\delta^{18}O$  values in 2014 than those at the CS. Variation in  $\delta^{18}O$  can be explained by environmental conditions (e.g., source water and relative humidity) and leaf physiological traits (e.g.,  $g_s$  and evaporation) (Roden and Ehleringer 2000, Barbour 2007). However, we can rule out differences in source water and microclimate as major drivers behind the observed differences in  $\delta^{18}O$  because BS and CS trees were growing in the same stand and experiencing similar environmental conditions over time (as suggested by similar  $\delta^{18}O$  values in the period before PB). The trees sampled were similar in age and size at the time of the study. Therefore, it is unlikely that unburned and burned trees were accessing water at different depths (Brooks et al. 2006). Furthermore, it could be argued that the decreased  $\delta^{18}O$  values in PB years could be linked to less enriched source water at greater depth due to significant mortality of shallow roots after PB. However, root injury is rarely described as a consequence of PB (Swezy and Agee 1991, Zeleznik and Dickmann 2004, Hood et al. 2010, Noonan-Wright et al. 2010), and *P. pinea* is a species with an extensive root system (Mazza et al. 2014), sparse in the first 15 cm of soils while increasing below 20 cm (Frattegianni et al. 1994).

Lower  $\delta^{18}O$  in PB stand suggests that the reduction in herb and shrub competition for soil resources enhanced the water status of the dominant trees. *Pinus pinea* is a drought-avoiding species with tight stomatal control of transpiration and photosynthesis under water-limiting conditions (Oliveras et al. 2003). Lower  $\delta^{18}O$  suggests higher leaf-level  $g_s$  in BS than in CS after PB (Barbour 2007). Whereas  $\delta^{18}O$  in the period before PB (2005–13) was indistinguishable between sites assigned to different treatments, significant differences were found in 2014, thus indicating a short response time (a few months) of tree-ring  $\delta^{18}O$  to differences in competition intensity in *P. pinea*. On the contrary, tree-ring  $\delta^{13}C$  did not differ between treatments, suggesting that time-integrated  $WUE_i$  in the remaining trees was unaffected by fire. A similar study carried out at a PB site in southern Italy on *P. halepensis* showed different plant responses in old dominant trees and the youngest individuals (Battipaglia

et al. 2014b). At burned sites, variation in productivity and  $WUE_i$  was mostly related to an increase in photosynthetic activity, probably due to post-fire fertilization effects, while  $g_s$  did not change significantly (Battipaglia et al. 2014b). On the contrary, the young individuals showed an increase in both  $\delta^{13}C$  and  $\delta^{18}O$ , suggesting that  $g_s$  was the dominant limitation to photosynthesis (Battipaglia et al. 2014b).

In the present study, the lack of response in terms of  $\delta^{13}C$ , combined with lower  $\delta^{18}O$  at BS, should be related to a parallel increase of similar magnitude in both  $A$  and  $g_s$  (Scheidegger et al. 2000, Grams et al. 2007). Indeed, fire can reduce competition from the understory vegetation (Grimsson 1985, Rozas et al. 2011), changing not only the soil water regime (Pinto 1990, Rego and Rigolot 1990, Rego and Botelho 1992) but also nutrient availability (Beghin et al. 2011, Renninger et al. 2013). Several studies focused on forest thinning have shown the same isotopic results with an enhanced  $A$  and  $g_s$  in the remaining trees as a consequence of a reduction in stand competition (Sala et al. 2005, McDowell et al. 2006, Moreno-Gutiérrez et al. 2011). Also, the strong correlations of tree-ring  $\delta^{13}C$  and both  $\delta^{18}O$  and TRW across individuals of both burned and unburned sites indicate that  $WUE_i$  and productivity of *P. pinea* are largely regulated by changes in stomatal aperture.

### Conclusion

There is an intense debate on the consequences of PB on the different ecosystem components. In this study, for the first time to our knowledge, we assessed various aspects of the physiological responses of adult trees to PB. Classical dendrochronological approaches were linked to anatomical, hydraulic and isotopic analyses to assess *P. pinea* responses to PB and to highlight the possible interaction of PB with climate and drought in the Mediterranean ecosystem.

Our findings demonstrate that PB has no effects on eco-physiology and stem hydraulics of *P. pinea*, a critical aspect for trees in the drought-prone Mediterranean environment. The post-fire decrease of  $\delta^{18}O$  reflected higher  $g_s$  and higher photosynthetic rate of burned trees in comparison with control trees, due to decreased competition for water. More significant effects on tree physiological processes might be expected upon exposure to higher burn severity by increasing the fire intensity. However, this would be poorly representative of the management burns usually implemented in these forest ecosystems (Fernandes et al. 2013). The long-term effect of the PB described in this study will be monitored constantly in the future, and interaction with drought, to which the species is highly sensitive, will be further explored. However, the fact that  $g_s$ , assessed through tree-ring  $\delta^{18}O$ , has a short response time to PB in comparison with the other parameters may be an important consideration for managers interested in assessing the effectiveness of PB practices and for modelers who are

interested in predicting the interaction of PB with fluctuation in the precipitation regime.

## Supplementary data

Supplementary data for this article are available at *Tree Physiology* Online.

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## Conflict of interest

None declared.

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