Drought-induced dieback of *Pinus nigra*: a tale of hydraulic failure and carbon starvation

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Ongoing climate change is apparently increasing tree mortality rates, and understanding mechanisms of drought-induced tree decline can improve mortality projections. Differential drought impact on conspecific individuals within a population has been reported, but no clear mechanistic explanation for this pattern has emerged. Following a severe drought (summer 2012), we monitored over a 3-year period healthy (H) and declining (D) *Pinus nigra* trees co-occurring in a karstic woodland to highlight eventual individual-specific physiological differences underlying differential canopy dieback. We investigated differences in water and carbon metabolism, and xylem anatomy as a function of crown health status, as well as eventual genotypic basis of contrasting drought responses. H and D trees exploited the same water pools and relied on similar hydraulic strategies to cope with drought stress. Genetic analyses did not highlight differences between groups in terms of geographical provenance. Hydraulic and anatomical analyses showed conflicting results. The hydraulic tracheid diameter and theoretical hydraulic conductivity were similar, but D trees were characterized by lower water transport efficiency, greater vulnerability to xylem conduit implosion and reduced carbohydrate stores. Our results suggest that extreme drought events can have different impacts on conspecific individuals, with differential vulnerability to xylem embolism likely playing a major role in setting the fate of trees under climate change.

**Key words:** Black pine, carbon metabolism, drought, plastome, rooting depth, water status

**Editor:** Kevin Hultine

Received 5 December 2018; Revised 22 January 2019; Editorial Decision 28 February 2019; Accepted 18 March 2019


***Introduction***

Recent trends towards more frequent anomalous heat and drought waves in broad areas around the world have been suggested to arise from ongoing climate warming (Kumar *et al*., 2013) and have already caused vegetation shifts and forest decline in several biomes (Mueller *et al*., 2005; Allen *et al*., 2010; Nardini *et al*., 2013; Gaylord *et al*., 2015; Hember *et al*., 2017). Considering that climate models forecast increasing intensity/severity of extreme drought events...
over the next decades, there is an increasing concern about potential impacts on tree species’ survival, regional land cover, ecosystem services and functions.

Heat and drought negatively influence vegetation through effects on water and carbon metabolism (McDowell et al., 2011; Reinhardt et al., 2015; Salmon et al., 2015; Savi et al., 2016), and water stress is a common cause of tree decline (Anderegg et al., 2013; Nardini et al., 2014a). As soil water availability decreases, water potential in xylem conduits (Ψ\text{xylem}) decreases (Tyree and Zimmermann, 2002), reducing the safety margins against embolism formation (Markesteijn et al., 2011; Johnson et al., 2012). When species-specific critical Ψ\text{xylem} thresholds are surpassed, the capacity of the xylem to supply water becomes insufficient to sustain transpiration (Tyree and Ewers, 1991), and trees may eventually succumb to catastrophic hydraulic failure (Nardini et al., 2013).

Heat and drought may also impact the carbon status of trees (Galiano et al., 2012; Poyatos et al., 2013), as the rate of non-structural carbohydrate (NSC) consumption for metabolism maintenance is correlated with temperature and water status (Amthor, 2000). Furthermore, low water availability leads to stomatal closure and reduced CO₂ uptake which, coupled to depletion of NSC, can induce negative carbon balance and ‘starvation’ (Allen et al., 2010; Sevanto et al., 2014; McDowell et al., 2016; Nardini et al., 2016). Chronic impairment of water transport and carbon balance reduces tree vigor, predisposing plants to biotic attacks that may further impact tree health (Aguadé et al., 2015).

Recent experiments have addressed drought-induced tree decline by considering hydraulic failure and carbon starvation as interconnected processes (Aguadé et al., 2015; Nardini et al., 2016; Savi et al., 2016; Yoshimura et al., 2016; Petrucco et al., 2017; Tomasella et al., 2017). It has been suggested that species with a more isohydric response to drought (stomatal control, avoidance of low Ψ\text{xylem} conserving water use) might be more exposed to the risk of carbon starvation, while anisohydric plants (maintenance of gas exchange, tolerance of low Ψ\text{xylem}) can succumb to extreme water shortage because of hydraulic failure (Allen et al., 2010; Nardini et al., 2014a). However, woody plants exhibit a continuum of hydraulic strategies, rather than a clear distinction between two contrasting alternatives (Johnson et al., 2012; Adhikari and White, 2014; Sevanto et al., 2014). Furthermore, intraspecific variability in water-use strategies and drought response is not negligible (Poyatos et al., 2013; Hentschel et al., 2014; Nardini et al., 2014b; García-Forner et al., 2016; Petrucco et al., 2017). With retrospective measurements on mature trees after an extreme drought, Gaylord et al. (2015) found a significantly greater frequency of aspirated tracheid pits in dead Pinus edulis trees compared to living ones, suggesting their higher vulnerability to embolism formation. Moreover, a significant difference in wood δ¹³C between health classes suggested lower stomatal conductance and greater carbon limitation in declining trees (Gaylord et al., 2015). Opposite trends were found by Salmon et al. (2015), reporting higher stomatal conductance, transpiration and photosynthetic rates in declining individuals of Pinus sylvestris compared to symptom-less trees. However, despite the relatively anisohydric and isohydric response to drought of defoliated and healthy individuals, respectively, few differences were observed in NSC content (Salmon et al., 2015). On the other hand, no differences in plant water status and vulnerability to embolism were observed when comparing healthy and declining Scots pine during a dry and warm summer. However, defoliated trees had lower NSC content before, during and after drought in both above- and below-ground organs (Aguadé et al., 2015). Overall, intra-population differences in drought-induced tree mortality are difficult to interpret due to possible phenotypic differences in physiological responses, anatomical adaptations and genetic variability of different conspecific individuals (Cercuera et al., 2011; López et al., 2016), coupled to complex microclimatic and edaphic gradients.

The unusually warm spring–summer droughts that occurred in 2003 and 2012 in southern Europe led to region-wide forest decline (Rebetez et al., 2006; Venetier et al., 2007; Nardini et al., 2013; Aguadé et al., 2015). Petrucco et al. (2017) reported widespread dieback of Pinus nigra in a karstic region characterized by shallow and highly permeable soils, apparently triggered by the anomalous drought that occurred in the area in 2012 (Nardini et al., 2013). Starting from winter–spring 2013, declining and symptom-less individuals were standing next to each other. Dendrochronological measurements coupled with tree-rang carbon and oxygen isotopic composition analysis in differentially affected trees revealed that declining trees had higher growth rates than healthy ones at the beginning of the 20th century, but their growth was reduced after the dry summers of 2003 and 2012. Moreover, results suggested that the two groups adopted different hydraulic strategies under drought, i.e. preferentially isohydric and anisohydric in healthy and declining individuals, respectively (Petrucco et al., 2017).

Here, we report measurements of water status and carbon stores of P. nigra trees in the years following the extreme drought of 2012. We aimed to investigate post-drought legacies on water and carbon metabolism of declining and healthy trees, as well as possible differences in rooting patterns and/or exploitation of soil water. Hydraulic measurements and anatomical analyses of stem segments were used to highlight different hydraulic efficiency/safety. Based on Petrucco et al. (2017), we also hypothesized declining trees to show a more anisohydric strategy and reduced carbon reserves. Finally, we hypothesized that different drought responses might arise from genetic differences due to possible different provenances of seeds used for reforestation.
Materials and methods

Study site and plant material

The study was carried out in the Bosco Bazzoni woodland (Trieste, 45° 37.8' N, 13° 51.7' E, 380 m a.s.l.). The site is located in the Classic Karst, the highly permeable limestone plateau extending across southwestern Slovenia and northeastern Italy. The pine woodland originated from repeated reforestation activities dating back since late 1800s (Sfregola, 2017). The dominant tree species is *P. nigra* J.F. Arnold subsp. *nigra*, while the understory comprises small-sized trees and shrubs like *Cotinus coggygria* Scop., *Fraxinus ornus* L. and *Prunus mahaleb* L. *P. nigra* (Black pine) is a sun-loving species growing on limestone cliffs, and it has been largely exploited for reforestation of arid and rocky habitats (Tutin et al., 1964). The climate of the study area is transitional between sub-Mediterranean and prealpine continental. Average annual temperature and rainfalls are ≈12.9°C and 1385 mm, respectively (www.osmer.fvg.it; 1992–2016). In 2003 and 2012 the area experienced anomalous summer drought and heat waves. In both episodes, air temperatures in August averaged ≈26°C vs 22.5°C of the reference mean, while precipitations during January–August were ≈50% less than the average.

Measurements were performed over the period 2014–2016 on five individuals showing dieback and desiccation in more than 50% of their crown (D), as visually estimated by four different observers to reduce possible subjective errors. These were compared to five individuals with still green and apparently healthy crowns (H). D and H trees (height of ∼10 m, 30–50 years old) were intermixed with no apparent spatial pattern. Samples collection and measurements were performed on south-exposed branches, at breast height. For genetic analyses (see below), a higher number of D and H trees was sampled.

Hydraulic measurements

To verify possible differences in terms of stem hydraulic conductivity and resistance to drought-induced xylem dysfunction, in spring 2014 hydraulic vulnerability curves (VCs) were measured with the bench dehydration technique. About 1.5 m long branches were collected from five trees per group early in the morning, re-cut under water, covered with a plastic bag, transported to the laboratory and rehydrated with their cut end immersed in water to favour rehydration (Trifiliò et al., 2014). Branches sampled from D trees had no dieback, but chlorotic needles. After 24 h, branches were left air dehydrating in the laboratory. At different time intervals, three apical shoots were wrapped in cling film and the branch inserted in a plastic bag. After 30 min of equilibration, $\Psi_{xyl}$ was measured on detached brachiablasts with a pressure chamber (mod. 150S5D, PMS Instruments, OR, USA). A stem segment (∼6 years old) was re-cut under water by progressively trimming 2 cm slices at both sides, until obtaining a 4 cm long segment (Venturas et al., 2015). Segments were debarked, trimmed and connected to a hydraulic apparatus (Xyl'em, Bronkhorst, Montigny-Les-Corneilles, France). A low pressure perfusion ($P = 6$ kPa) with a 10 mM KCl solution allowed to measure native sample hydraulic conductance ($K_s$). The length ($l$) and transverse xylem area ($A_{xyl}$) of the segment were measured and stem-specific hydraulic conductivity ($K_s$) was calculated as ($K_s l)/A_{xyl}$, Hence, plots of $K_s$ vs $\Psi_{xyl}$ (VCs) were constructed (Hacke et al., 2015; Schreiber et al., 2016) and a sigmoidal curve model was fitted to the data. Native embolism was not removed, because flushing samples at high pressure resulted in no change or decreased $K_s$, as also reported by Froux et al. (2002).

Additional experiments were carried out in spring 2016, when relative water loss (RWL) curves of five branches per group (see above) were measured (Rosner et al., 2008). Branches were re-cut to a final length of 15 cm, debarked and saturated in distilled and degassed water under partial vacuum for 24 h. After determination of saturated mass (SM), overpressure was applied ($P = 0.5$ MPa, 1 min) to the sample inserted in a double-ended pressure chamber (PMS Instruments, OR, USA) and the stem re-weighed (FM). The treatment was repeated by increasing $P$ in steps of 0.5 or 1 MPa until maximum $P = 6$ MPa. The samples were oven-dried at 103°C, their DM recorded and RWL calculated as $1 - ((FM - DM)/(SM - DM)) \times 100$.

Anatomical measurements and calculation of theoretical hydraulic conductivity

In spring 2014, 10 branches were sampled from H and D individuals and stem segments, similar to those used for hydraulic analyses, were prepared. These samples were used to measure basic wood density ($D_w$) as a proxy for drought tolerance (Rosner et al., 2008; Markesteijn et al., 2011; Trifiliò et al., 2015). Anatomical analyses were also performed to highlight differences between experimental groups related to post-drought health status. The anatomical characteristics of annual rings formed before the drought event, i.e. under sufficient water supply, were expected to provide information on potential predisposition to drought sensitivity (Rosner et al., 2016).

Stem segments were rehydrated overnight, debarked and their fresh volume ($V$) was measured (Hughes, 2005). Sample mass (DM) was obtained after complete drying (24 h at 103°C), and $D_w$ was calculated as DM/V. Samples were softened in a glycerin–ethanol-distilled water solution (1:1:1). After 4 days, 20 μm thick stem cross sections were obtained using a sliding microtome (Reichert-Jung, Optische Werke AG Wien, Austria) and stained with safranin. Images were acquired with a digital camera (Leica DFC 290, Wetzlar, Germany) connected to a microscope (Leica DM 5500B, Wetzlar, Germany) and analysed with ImageJ (https://imagej.nih.gov/ij/). Considering the last three complete annual rings (2011–2013), the following parameters were quantified: ring width and annual ring area, percentage of latewood, tracheid...
diameter (d), tracheid double wall thickness (t), the square of the thickness-to-span ratio (t/b)², tracheid density (Tₐ) and the hydraulic mean diameter (Dₕ), calculated as (i) \( \Sigma d^4/\Sigma d^4 \) (Kolb and Sperry, 1999; Hacke et al., 2001; Petit et al., 2016) and (ii) \( (\Sigma d^4/N)^{0.25} \) (Tyree and Zimmermann, 2002; Scholtz et al., 2013), when N is the total number of conduits measured. About 200 individual tracheids were measured on each branch. Using Dₕ calculated as suggested by Tyree and Zimmermann (2002) in the Hagen–Poiseuille equation, an estimate of hydraulic conductivity (Kₘₜ) for each growth ring was calculated according to Gonzalez-Benecke et al. (2010) as \( Tₐ \times \pi \times Dₕ^4/128 \eta \), where \( \eta \) is the viscosity of water (8.9 × 10⁻¹⁰ MPa s).

**Monitoring of plant water status and hydraulic strategies**

To highlight possible differences in water status between H and D trees, leaf water potential and conductance to water vapour (gL) were measured on two sunny days in spring (June) and summer (July) 2015. Pre-dawn (Ψₚd) and minimum (Ψₘₚ) water potential were measured on brachiblasts detached at 0.00 am and 13.00 am, respectively, from each of the 10 individuals. Samples were wrapped in cling film, transported to the laboratory in a cool bag and measured with the pressure chamber within 2 h from sampling. At midday, gL was measured on at least two brachiblasts per individual with a porometer (LI-1600, Li-Cor Inc., NE, USA). During all measurements, ambient air temperature (Tₐ, 25.2 ± 0.1 and 38.3 ± 0.3°C in June and July, respectively), relative humidity (RH, 22.6 ± 0.2 and 20.4 ± 0.3%) and photosynthetic photon flux density (PPFD, 1562 ± 80 and 1581 ± 45 μmol m⁻² s⁻¹) were recorded. Maximum vapour pressure deficit (VPD) was 2.5 kPa and 5.4 kPa in spring and summer, respectively. To check for eventual differences in stomatal responses to water stress in H and D trees (anisohydry vs isohydry), oxygen isotopic composition of xylem sap (δ¹⁸O) of samples was measured with an isotope ratio mass spectrometer (Delta Plus Advantage, Thermo Fisher Scientific, MA, USA; see also Nardini et al., 2016).

**NSC contents**

To highlight eventual differences in post-drought NSC reserves, soluble sugars (glucose, fructose and sucrose) and starch concentrations were measured on 3- to 5-year-old stem segments detached from H and D trees (one stem segment per tree, five trees per group) on the dates selected for water status measurements. Samples were transported to the laboratory in a cool bag, microwaved (700 W, 3 min; within 1 h after sampling), oven-dried at 70°C and kept frozen until analysis. Samples were pulverized (particle size <0.15 mm), dividing bark and wood, and 15 ± 1 mg of material was transferred in a 1.5 ml Eppendorf vial. NSC extraction and analysis followed the enzymatic method standardized by Quentin et al. (2015) adapted to low amounts of material (Savi et al., 2016). Samples were suspended in 1 ml of 80% ethanol solution for three times, the supernatant was used for soluble sugars measurement, while the pellet was re-suspended in 1 ml of Acetate buffer (0.4 M NaCH₃COO, pH = 4.6) and directed to starch evaluation. For glucose measurement, 5–20 ml of supernatant were transferred in a cuvette with 2 ml final volume of essay buffer solution solution (Tris-HCl with MgCl₂ 5 mM, NADP⁺ 125 μM and MgATP 1 mM, at 25°C) and placed in a spectrofluorimeter (LS50B Luminescence Spectometer, Perkin-Elmer, MA, USA). The reaction was conducted by adding 2 U of both glucose-6-phosphate dehydrogenase and hexokinase. When the enzymatic kinetic due to gluconolactone production reached steady state, the evaluation of fructose was obtained adding in the same cuvette 3 U of phosphoglucose isomerase, to convert fructose-6-phosphate produced with hexokinase in glucose-6-phosphate. For sucrose analysis, 100 ml of the supernatant were placed in a 1.5 ml Eppendorf tube with 300 ml of acetate buffer with of 25 U of invertase to break down sucrose into fructose and glucose. The tubes were kept at 55°C for 30 min, then 20 ml were processed as described above. For starch digestion we performed an overnight procedure at 55°C using 100 U of α-amylase and 25 U of amyloglucosidase per sample. To prevent further degradation, the samples were boiled for 3 min. For analyses, 10 μl of final supernatant were transferred in a cuvette with 2 ml final volume of essay
buffer. The starch digestion and spectrofluorimeter analysis was also performed with known amounts of Amylose to obtain a calibration curve. The final concentration of starch in the sample was then expressed as % dry mass (% DM).

**Genetic analysis**

Because the pine woodland under study results from reforestation activities, we verified the eventual genotypic basis for different responses to drought between D and H trees, as a possible consequence of different seeds provenances. Three chloroplast microsatellites loci [simple sequence repeats (SSRs)] were analysed (Naydenov et al., 2006). In April 2015, green needles of 50 trees per experimental category were collected from trees belonging to different age classes (20–100 years old), grinded in liquid nitrogen and stored frozen. Total DNA was extracted with the E.Z.N.A. kit (Plant DNA kit, Omega Bio-tek Inc, Norcross, GA, USA), quantified with a spectrophotometer (NanoDrop, Thermo Fisher Scientific, MA, USA) and three plastome microsatellites loci (Pt30204, Pt71936, Pt45002) were amplified with fluorescently labelled primers (Naydenov et al., 2006). Amplicons were resolved on agarose gel to verify amplification efficiency and quality and finally molecular weights were analysed using the ABI 3130 capillary sequencer with a ROX-labelled size standard (ABI 3130 Genetic Analyzer, Applied Biosystem, CA, USA). As a control, the same loci were also analysed in needles of three individuals of *Pinus halepensis*.

**Statistical analysis**

Data were analysed using SigmaStat v. 2.03 (SPSS Inc.) and R (R i386 3.2.5). Data normality and homoscedascity were assessed and statistically significant differences were highlighted by Student’s *t*-test, two- and three-way ANOVA (analysis of variance) (*P* < 0.05). An ANCOVA (analysis of covariance) was applied to test differences in the response to drought of H and D trees. The SSRs data were analysed with the software STRUCTURE. The most likely number of clusters (K) was estimated using the complementary software Structure Harvester v 0.6.94 (Pritchard et al., 2000).

**Results**

Hydraulic VCs of D and H trees (Fig. 1) were based on 71 and 74 hydraulic measurements, respectively, and the reference parameters *P*$_{20}$ and *P*$_{50}$ (*Ψ$_{xylem}$* inducing 20 and 50% loss of *K*$_{s}$) were derived from VCs using the r-package fit-PLC (Duursma and Choat, 2016). The *P*$_{20}$ resulted slightly more negative in H than in D individuals (−1.7 vs 1.4 MPa), while *P*$_{50}$ was lower in D ones (D = −3.6 MPa, H = −3.2 MPa). However, both parameters were not significantly different between the two groups of trees, as indicated by the overlapping 95% confidence intervals (Table S1). The maximum stem-specific hydraulic conductivity (*K*$_{max}$), calculated as the average *K*$_{s}$ at *Ψ$_{xylem}$* > −0.5 MPa, was significantly lower (*P* < 0.001) in D trees (0.52 ± 0.04 kg s$^{-1}$ MPa$^{-1}$ m$^{-1}$) than in H ones (0.72 ± 0.03 kg s$^{-1}$ MPa$^{-1}$ m$^{-1}$). The inset in Fig. 1 reports the VC of H trees based on *K*$_{s}$ values within the observable range for both populations (0.03–0.68 kg s$^{-1}$ MPa$^{-1}$ m$^{-1}$).

![Figure 1: Vulnerability curves (VCs) reporting the relationship between stem-specific hydraulic conductivity (Ks) and xylem water potential (Ψxylem), as measured for healthy (H, closed circles, solid line) and desiccated (D, open circles, dashed line) *P. nigra* trees. The sigmoidal regressions are also reported. The Ψxylem inducing 20 (P20) and 50 (P50) % loss of Ks were −1.67 and −3.24 MPa, and −1.42 and −3.63 MPa for H and D group, respectively (fit-PLC, Duursma and Choat, 2016). The insets show the VC of H trees based on Ks data that were within the observable range for both populations (0.03–0.68 kg s$^{-1}$ MPa$^{-1}$ m$^{-1}$).](https://academic.oup.com/conphys/article-abstract/7/1/coz012/5485269)

In spring and summer, under respectively high and low soil water availability, Ψ$_{pd}$ averaged −0.45 MPa and −1.37 MPa...
Table 1: Ring width, annual ring area, percentage of latewood, tracheid diameter (d), tracheid wall thickness (t), thickness-to-span ratio, tracheid density, hydraulic mean diameters ($D_h$) and theoretical hydraulic conductivity ($K_{theor}$) measured for healthy (H) and desiccated (D) individuals in the last tree annual rings (2011–2013)

<table>
<thead>
<tr>
<th></th>
<th>Healthy</th>
<th>Desiccated</th>
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<tbody>
<tr>
<td>Ring width, mm</td>
<td>0.29 ± 0.05 $^a$</td>
<td>0.20 ± 0.04 $^ab$</td>
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<tr>
<td>Ring area, mm$^2$</td>
<td>4.6 ± 0.7</td>
<td>3.3 ± 0.9</td>
</tr>
<tr>
<td>Late wood, %</td>
<td>27.7 ± 4.4</td>
<td>24.1 ± 5.2</td>
</tr>
<tr>
<td>Tracheid diameter, μm</td>
<td>12.0 ± 0.4</td>
<td>12.4 ± 0.3</td>
</tr>
<tr>
<td>Wall thickness, μm</td>
<td>4.0 ± 0.3</td>
<td>3.8 ± 0.2</td>
</tr>
<tr>
<td>(t/b)$^2$</td>
<td>0.19 ± 0.03 $^a$</td>
<td>0.17 ± 0.01 $^a$</td>
</tr>
<tr>
<td>Tracheid density, mm$^{-2}$</td>
<td>3739 ± 83</td>
<td>3687 ± 231</td>
</tr>
<tr>
<td>$D_h$ (Kolb and Sperry, 1999), μm</td>
<td>15.7 ± 0.6</td>
<td>16.3 ± 0.3</td>
</tr>
<tr>
<td>$D_h$ (Tyree and Zimmermann, 2002), μm</td>
<td>13.2 ± 0.4</td>
<td>13.8 ± 0.2</td>
</tr>
<tr>
<td>$K_{theor}$, kg s$^{-1}$ MPa$^{-1}$ m$^{-1}$</td>
<td>3.2 ± 0.2</td>
<td>3.7 ± 0.1</td>
</tr>
</tbody>
</table>

Values with significant differences are reported in bold. Mean ± SEM are reported. Upper-case letters indicate statistically significant difference ($P < 0.05$) between health classes (Factor I), while lower-case letters indicate statistically significant difference among years (Factor II), as tested using two-way ANOVA. No statistically significant interaction between factors was observed.

Table 2: Pre-dawn ($\Psi_{pd}$) and minimum water potential ($\Psi_{min}$) and leaf conductance to water vapour ($g_L$) measured in healthy (H) and desiccated (D) trees in June (a) and July (b) 2015

<table>
<thead>
<tr>
<th></th>
<th>Healthy</th>
<th>Desiccated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>17 June 2015</td>
<td></td>
</tr>
<tr>
<td>$\Psi_{pd}$, −MPa</td>
<td>0.44 ± 0.01</td>
<td>0.49 ± 0.04</td>
</tr>
<tr>
<td>$\Psi_{min}$, −MPa</td>
<td>0.96 ± 0.03</td>
<td>1.08 ± 0.06</td>
</tr>
<tr>
<td>$g_L$, mmol m$^{-2}$ s$^{-1}$</td>
<td>160.2 ± 12.8</td>
<td>213.2 ± 25.7</td>
</tr>
</tbody>
</table>

Mean ± SEM are reported.

in both experimental groups (Table 2). The $\Psi_{min}$ dropped to about −1.00 MPa in spring and reached −1.70 MPa in summer. A non-significant ($P = 0.1$) trend towards more negative $\Psi_{min}$ in D than in H trees was observed, consistent with ~30% higher $g_L$ values measured in spring in the former group compared to the latter (213 ± 26 vs 160 ± 13 mmol m$^{-2}$ s$^{-1}$, $P = 0.1$).

Figure 2 reports changes in relative leaf conductance to water vapour ($g_{L\ REL}$) measured in H and D branches as a function of $\Psi_{leaf}$. D and H trees did not differ in terms of maximum $g_L$ measured in fully hydrated branches exposed to sun, which averaged 433 ± 30 mmol m$^{-2}$ s$^{-1}$. The ANCOVA test did not highlight statistically significant differences in the response of $g_L$ to $\Psi_{leaf}$ between the two categories.

The oxygen isotopic composition of xylem sap extracted from H and D stems in June averaged −8.0 ± 0.3‰ and −7.7 ± 0.2‰, respectively (Fig. 3). In July, the $\delta^{18}$O signifi-
Genetic polymorphism data from three chloroplast microsatellites loci were obtained from 50 different individuals per experimental group (Table S3). The size range for the loci Pt30204, Pt71936 and Pt45002, respectively, i.e. very similar to those found in the reference paper for the same species, was 136–142 bp, 142–146 bp and 162–165 bp for the loci Pt30204, Pt71936 and Pt45002, respectively. Using STRUCTURE analysis, we estimated the potential number of populations (K) to which the individuals could be attributed, without being assigned a priori to hypothetical populations. The analysis indicated that the probability of K = 1 was greater than that of K = 2. To validate the hypothesis that there is no population structure, we further performed a supervised clustering with K = 2. Figure S2 shows the probability of assignment of each individual to two putative populations.

Discussion

Anomalous drought and heat waves have produced large-scale impacts on forest ecosystems (Matusick et al., 2013; Aguadé et al., 2015; Hember et al., 2017). One year after the 2012 severe drought, several Black pines suffered extensive crown dieback. On the basis of visual assessments performed in spring 2014, 2015 and 2016 no signs of recovery could be detected in D individuals, while H plants showed still completely healthy crowns. Our retrospective analyses revealed both similarities and subtle differences between declining and symptom-less trees.

The analysis of VCs yielded a P50 of about −3.2 MPa for H trees, in accordance with published values for the same species (Froux et al., 2002; Martínez-Vilalta and Piñol, 2002; Hacke et al., 2001) and within the range reported for other Pinus species (P50 varying from −2.3 MPa to −7.0 MPa). Hence, Black pine appears as a species relatively vulnerable to drought-induced xylem dysfunction (Hacke et al., 2001; Lamy et al., 2011; Sáenz-Romero et al., 2013; Battipaglia et al., 2016). Hydraulic VCs also suggested a slightly higher resistance against drought-induced embolism of D individuals, which displayed ∼0.4 MPa lower (more negative) P50 than that recorded in H trees. Although not significant, this is an opposite trend compared to data reported in recent studies (Anderegg et al., 2013; Nardini et al., 2014b). While the native hydraulic conductivity (Ks) of H trees was in agreement with previously published values (Froux et al., 2002), D trees showed a significantly lower Ks, as a possible consequence of embolism accumulated during the drought period. In fact, when the VC of H trees was recalculated based only on values of Ks that were within the observable range for D trees, the interpolated P50 was nearly identical in the two groups (see inset in Fig. 1).

Plant hydraulic performance is influenced by xylem anatomy (Hacke et al., 2001; Tyree and Zimmermann, 2002; Scholz et al., 2013; Petit et al., 2016), with wider conduits leading to higher Ks. In particular, hydraulically weighted tracheid diameter (Tyree and Zimmermann, 2002) is linearly related to Kmax in pine species (Gonzalez-Benecke et al., 2010). Interestingly, we did not observe anatomical differences between D and H trees (Table 1) that could account for recorded differences in Ks. Ring width and area, tracheid diameter, hydraulic diameters, as well as theoretical hydraulic conductivity, were similar in H and D individuals in the outermost annual rings. H individuals had (t/b)2 ∼30%
Figure 4: Glucose (a), fructose (b), sucrose (c) and starch (d) concentration measured in bark and wood of H (black and grey dashed columns, respectively) and D (white and white dashed columns, respectively) trees in June and July 2015. Mean ± SEM are reported. Upper-case letters and asterisks indicate statistically significant difference (P < 0.05) between health classes (Factor I) and growing seasons (Factor III), respectively. For sucrose and starch statistically significant differences between wood and bark tissue (Factor II) was also observed. Complete output of the three-way ANOVA in Table S2.

higher than D ones (0.17 ± 0.01 vs 0.13 ± 0.01, pooled for the three outermost rings), suggesting higher conduit reinforcement and consequently higher safety towards tracheid collapse under negative pressure in H trees. Since (t/b)² has been correlated with the resistance against embolism formation (Hacke et al., 2001; Willson and Jackson, 2006; Rosner et al., 2016), this finding suggests that D trees were more vulnerable to drought-induced loss of water transport efficiency. This is also supported by RWL curves, showing that D trees have a less negative xylem pressure threshold inducing embolism formation compared to H trees and lose significantly larger water volumes at similar $\Psi_{\text{xyl}}$ (Fig. S1; Table S1). The overpressure resulting in 50% RWL is considered a good proxy for hydraulic vulnerability (Rosner et al., 2008). In our study, $\Psi_{\text{RWL50}}$ was reached at very high overpressure (~6 MPa) but, interestingly, interpolating $\Psi_{\text{RWL30}}$ from the fitted models resulted in a difference of 0.6 MPa between D and H trees (3.54 MPa vs 4.14 MPa, respectively).

Overall, the results of hydraulic and anatomical analyses suggest that the observed difference in terms of $K_s$ was a legacy of the drought event that produced a differential impact on the two groups of trees, so that tracheids of D stems were partially embolized and not conductive when hydraulic measurements were performed. This residual embolism level was apparently not recovered during night-time branch rehydration prior to hydraulic measurements. As a consequence, the $K_s$ of D trees was significantly lower at any given $\Psi_{\text{xyl}}$ and the VC was ‘shifted’ towards more negative $\Psi_{\text{xyl}}$ values with respect to that of H trees (Hacke et al., 2015). The reduced efficiency of water delivery to foliage, consequent to the accumulated embolism, might provide an explanation for the observed partial desiccation of the crown in D individuals.

The lower cell wall reinforcement of D trees revealed limited carbon investments in xylem safety in declining individuals, suggesting limitations in terms of NSC availability as a consequence of reduced photosynthetic area due to defoliation, stomatal aperture and limitation of carbon fixation (Poyatos et al., 2013). NSC analyses confirmed that carbon stores were reduced in D trees (Fig. 4; Table S2). The increase of glucose and fructose concentration observed in July in both groups suggests that H and D trees likely...
adopted the same strategy of NSC mobilization to cope with seasonal drought. The increase of soluble sugars can arise as a consequence of both photosynthetic fixation and degradation of NSC (consistent with observed starch depletion). Intriguingly, sucrose and starch showed a different behaviour in H and D plants with the latter displaying significantly lower (by \( \sim 50\%)\) starch concentration compared to the former. This difference was evident in spring, while values of starch concentration were more similar in summer, when the larger reserves in H trees had been depleted already. The significant drop in starch supports the hypothesis of its mobilization under drought, likely leading to higher sugar availability in H than in D trees (\( \Delta \) starch 5.37 and 1.19% DM, respectively) providing energy to support growth and metabolism. Higher concentrations of NSC in healthy pine trees compared to desiccated ones, and an increase of soluble sugars under drought have been already reported, suggesting that both defoliation and prolonged periods of near complete stomatal closure contribute to reduce NSC in trees (Galiano et al., 2012; Poyatos et al., 2013; Aguadé et al., 2015; Vilela et al., 2016).

Our data suggest that the decline of some \( P. \) nigra individuals arise as a consequence of xylem embolism triggered by drought stress, coupled to impending carbon starvation (Kono et al., 2019). Considering that the woodland under study results from reforestation, we hypothesized that the differential drought impact on individuals could be linked to different origins of seeds/seedlings used, also taking into account that \( P. \) nigra displays high genetic distance between populations (Thiel et al., 2012) and considering that provenance-based differences in xylem vulnerability have been reported for different \( P. \) species. However, genetic analysis focused on the plastome did not highlight the presence of distinct populations among sampled individuals. Our analysis was based on the study of only three microsatellites loci, and it was aimed at detecting eventual differences in the geographical origin of seeds used for plant production and afforestation in the late 1800s. On this basis, we cannot exclude the occurrence of other genotypic differences between H and D trees, which might eventually explain their different vulnerabilities and also represent an interesting starting basis for selection of \( P. \) nigra genotypes better adapted to future climate scenarios. Indeed, previous studies have shown that the variability of vulnerability to drought in different tree species can be larger within populations than between populations (Coccuera et al., 2011; Wortemann et al., 2011). In order to obtain conclusive evidence on this point in the case of \( P. \) nigra, a complete genetic analysis based on genome-wide scan methodologies would be needed to reveal differences between the two groups.

Isotopic analyses of xylem sap revealed that D and H trees exploited fundamentally the same water pool. In fact, \( ^{81} \)O was about \(-8\%\) in June and \(-6\%\) in July for both groups, suggesting lack of differences in rooting depth (Ehleringer and Dawson, 1992). This does not rule out the possibility that D trees have a less extensive root system or are deficient in some other way in their ability to access and absorb water. A comparison of our data with a recent study focused in an area located <1 km from the pine woodland (Nardini et al., 2016) suggests prevalent use of shallow water resources by Black pine in our experimental site.

A recent study by Petrucco et al. (2017), performed in the same study site, has reported lower values of \( ^{13} \)C in wood cores of D trees compared to H ones, suggesting delayed stomatal closure under drought and more pronounced water-saving behaviour in the former group. In our study, seasonal changes in \( \Psi_{\text{leaf}} \) and \( g_L \) indicated that D and H trees did not experience different levels of water stress during the normal seasonal drought that occurred in 2015. This is consistent with findings by Aguadé et al. (2015), reporting similar \( \Psi \) values in \( P. \) sylvestris affected by desiccation compared to still healthy individuals, but opposite to results of Hentschel et al. (2014) and Salmon et al. (2015). However, during spring we recorded a weak and marginally significant trend \( (P = 0.1) \) towards lower \( \Psi_{\text{min}} \) (by \( \sim 13\%\)) and higher \( g_L \) (by \( \sim 33\%) \) in D trees compared to H ones. These results would support the hypothesis that D trees display a more anisohydric response to drought (Petrucco et al., 2017). Considering the reduced photosynthetic area of D trees, their slightly more pronounced stomatal opening might also be interpreted as a compensatory physiological mechanism to maintain an overall positive carbon gain. Similarly, recent studies recorded significantly higher stomatal conductance and net \( CO_2 \) assimilation in defoliated than in non-defoliated Scots pines and Norway spruce, with the former group displaying also significantly lower \( \Psi_{\text{pd}} \) and \( \Psi_{\text{min}} \) (Hentschel et al., 2014; Salmon et al., 2015).

Opposite to previous findings highlighting different responses of canopy stomatal conductance to \( \Psi \) changes in defoliated and non-defoliated \( P. \) sylvestris (Poyatos et al., 2013), the \( g_L \) response to \( \Psi_{\text{leaf}} \) was very similar in H and D trees, suggesting that stomata responded similarly to leaf dehydration in the two groups. Stomata are known to respond to several different environmental and physiological factors, besides needle water status (Mott and Peak, 2011; McAdam and Brodribb, 2015). Hence, we cannot exclude different intrinsic responsiveness of stomata of H and D trees to other factors such as air temperature, RH, wind and irradiance. In particular, differences in \( g_L \) response to VPD at relatively constant \( \Psi_{\text{leaf}} \) values, not tested in our study, might provide an explanation for different hydraulic strategies in H and D trees (McAdam and Brodribb, 2015; Marchin et al., 2016).

Our study confirms that drought-induced canopy desiccation is a complex output of interactions between water and carbon metabolism. In fact, differences in the carbon status of \( P. \) nigra trees after a severe drought can apparently lead to subtle differences in the xylem structure and its efficiency, which may develop into declining tree vigor. These data, coupled to similar conclusions by recent studies (Galiano et al., 2012; Salmon et al., 2015), suggest that the analysis of species-
specific resistance against drought is not sufficient to fully understand and model the phenomenon of drought-induced tree die-off. The assessment of the risk of tree decline, and the development of quantitative and qualitative predictions of species abundance, diversity and richness under climate change scenario, will need to take into account that small and continuous variations in environmental and edaphic characteristics (Davi and Cailleret, 2017), as well as genotypic or phenotypic intraspecific variability (Taeger et al., 2013; Stojnić et al., 2017) can play fundamental roles in defining individual drought responses/impacts, dictating the fate of single trees under global-change-type droughts.

**Acknowledgements**

We are grateful to F. Polazzo, J. Luglio and E. Moretti for assistance during sampling and analysis.

**Funding**

The study was funded by the Fondazione Beneficentia Stiftung (Project title: Cambiamenti climatici e mortalità degli alberi: basi genotipiche della resistenza e resilienza a eventi estremi di aridità).

**Supplementary material**

Supplementary material is available at Conservation Physiology online.

**References**


