



## Research paper

# Diurnal changes in embolism rate in nine dry forest trees: relationships with species-specific xylem vulnerability, hydraulic strategy and wood traits

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Recent studies have reported correlations between stem sapwood capacitance ( $C_{\text{wood}}$ ) and xylem vulnerability to embolism, but it is unclear how  $C_{\text{wood}}$  relates to the eventual ability of plants to reverse embolism. We investigated possible functional links between embolism reversal efficiency,  $C_{\text{wood}}$ , wood density (WD), vulnerability to xylem embolism and hydraulic safety margins in nine woody species native to dry sclerophyllous forests with different degrees of iso versus anisohydry. Substantial inter-specific differences in terms of seasonal/diurnal changes of xylem and leaf water potential, maximum diurnal values of transpiration rate and xylem vulnerability to embolism formation were recorded. Significant diurnal changes in percentage loss of hydraulic conductivity (PLC) were recorded for five species. Significant correlations were recorded between diurnal PLC changes and  $P_{50}$  and  $P_{88}$  values (i.e., xylem pressure inducing 50 and 88% PLC, respectively) as well as between diurnal PLC changes and safety margins referenced to  $P_{50}$  and  $P_{88}$ . WD was linearly correlated with minimum diurnal leaf water potential, diurnal PLC changes and wood capacitance across all species. In contrast, significant relationships between  $P_{50}$ , safety margin values referenced to  $P_{50}$  and WD were recorded only for the isohydric species. Functional links between diurnal changes in PLC, hydraulic strategies and WD and  $C_{\text{wood}}$  are discussed.

**Keywords:** anisohydric, drought stress, embolism repair, isohydric,  $P_{50}$ ,  $P_{88}$ , safety margin, wood capacitance, wood density.

## Introduction

Long distance xylem water transport is driven by gradients in negative pressure along the root-to-leaf pathway (Dixon and Joly 1895). According to the cohesion–tension theory, water loss due to evaporative processes at the leaf level induces a decrease in the leaf water potential that is transmitted through the xylem network to the roots, where the pressure drop ultimately drives water uptake thus assuring water supply to leaves and maintenance of tissue hydration (Tyree and Zimmermann 2002, Wheeler and Stroock 2008). According to this widely accepted mechanism, water in the xylem conduits is maintained in the liquid phase while under negative pressure (tension), i.e., in a thermodynamically metastable state. Hence, xylem water is

prone to cavitation, i.e., a sudden transition to the vapor phase (Tyree and Zimmermann 2002). Spontaneous water cavitation is, however, unlikely to occur under pressures commonly recorded in the xylem (Zwieniecki and Secchi 2014). Rather, according to the air-seeding hypothesis, progressively lower xylem water pressure caused by a drop in soil water potential and/or high transpiration rates favors aspiration of air bubbles (embolisms) into functioning xylem conduits from nearby gas-filled compartments, through inter-conduit pit-membranes (Lens et al. 2013). Xylem embolism leads to the blockage of water flow through the conduits compromising the root-to-leaf water transport and plant carbon gain. The occasional occurrence of xylem embolism is not necessarily a catastrophic event for

plants, and it might even represent a useful hydraulic signal regulating stomatal aperture (Salleo et al. 2000, Trifilò et al. 2003, Tombesi et al. 2014). However, day-by-day accumulation of embolism during prolonged drought might lead to extensive xylem dysfunction, causing substantial reductions in photosynthetic productivity or even leading to plant desiccation and death (McDowell et al. 2011, Nardini et al. 2013). Moreover, accumulation of embolism could affect plant defensive ability in response to biotic attack (McDowell et al. 2011).

Plants vary widely in their vulnerability to xylem embolism, and these differences have been reported to be somehow related to xylem anatomy and drought-adaptation of different species (i.e., Sperry et al. 2006, Delzon et al. 2010, Lens et al. 2013). Strikingly, despite such differences, plants apparently operate at xylem pressures close to critical thresholds triggering embolism formation (Nardini and Salleo 2000, Meinzer et al. 2009, Choat et al. 2012). About 70% out of 226 forest species have been reported to experience seasonal minimum water potential values very close to those inducing 50% loss of xylem hydraulic conductivity ( $P_{50}$ ) (Choat et al. 2012). This convergence on a 'risky' hydraulic behavior, regardless of biome-specific rainfall regimes, was invoked as a factor portending increasing risks of tree decline and mortality under global change-type droughts (i.e., Hoffmann et al. 2011, Nardini et al. 2013).

Plants can cope with embolism-induced loss of hydraulic conductivity by seasonal or continuous production of new xylem conduits, a process which is effective only over medium to long term (Brodrribb et al. 2010). However, some plant species (Brodersen and McElrone 2013) have been reported to repair embolism and refill gas-filled vessels even when the xylem pressure in still functioning conduits is substantially negative ('novel refilling', Salleo et al. 1996, Bucci et al. 2003, Hacke and Sperry 2003, Nardini et al. 2008). Recent controversies have raised the doubt that some of the reports describing 'novel refilling' might have been biased by experimental artifacts (Wheeler et al. 2013, Cochard et al. 2015), but newly performed control experiments (Trifilò et al. 2014b, Scoffoni and Sack 2015, Venturas et al. 2015) as well as in vivo observations of xylem embolism formation and recovery (Brodersen et al. 2010, Zwieniecki et al. 2013, Knipfer et al. 2015) suggest that the phenomenon is not a mere technical pitfall of sampling and hydraulic techniques.

Although the details of embolism repair are largely unresolved, the process apparently relies on generation of local positive pressures via osmotic mechanisms triggered by solute release from vessel-associated parenchyma into the embolized conduit (Salleo et al. 2004, Secchi and Zwieniecki 2012). This would generate water inflow favored by aquaporins (Laur and Hacke 2013, Secchi and Zwieniecki 2014) and likely supported by phloem-derived water (Nardini et al. 2011, Mayr et al. 2014, Pfautsch et al. 2015). In this scenario, the presence/abundance of xylem parenchyma (Zheng and Martinez-Cabrera 2013) as well as functional traits of bark tissues (Rosell et al. 2014, Bloemen et al.

2015) apparently play key roles in determining the availability of solutes and/or water for the refilling process. Interestingly, recent studies have reported positive correlations between stem sapwood capacitance and vulnerability to embolism (Meinzer et al. 2009, McCulloh et al. 2014). Assuming that the amount of living cells has a direct effect on xylem capacitance, these relationships might suggest that species more vulnerable to xylem embolism are better equipped for active refilling of embolized conduits (Johnson et al. 2012). Indeed, in a recent paper Ogasa et al. (2013) showed that embolism reversal is more efficient in species with higher vulnerability to embolism, suggesting that refilling mechanisms might compensate for higher drought vulnerability in some woody species. Interestingly, the efficiency of embolism reversal across species was found to be inversely correlated to wood density (WD). Because WD is inversely correlated to wood capacitance (Richards et al. 2014), these findings might suggest direct functional links between embolism reversal efficiency and wood capacitance, opening interesting questions about possible relationships between species-specific vulnerability to xylem embolism, occurrence of refilling mechanisms, hydraulic strategies under drought stress and wood traits.

The present paper reports diurnal changes in percentage loss of hydraulic conductivity (PLC) in nine woody species occurring in the same drought-prone biome (dry sclerophyllous forests) but characterized by different hydraulic strategies in terms of varying degrees of isohydry/anisohydry. We selected species native to the Mediterranean biome, one of the most biodiversity-rich spots on the planet (Cowling et al. 1996), but also most exposed to potentially harmful effects of ongoing climate change (Martínez-Vilalta et al. 2002, Giorgi and Lionello 2008, Matusick et al. 2013, Nardini et al. 2014). We specifically checked possible correlations between: (i) xylem vulnerability to embolism and diurnal cycles of embolism formation/repair; (ii) embolism reversal efficiency and species-specific hydraulic safety margins; and (iii) embolism repair and species-specific WD and capacitance.

## Materials and methods

Experiments were performed between May and September 2013 on nine angiosperm woody species (see Table 1) growing at the campus of the Department of Biological Sciences and Environment, University of Messina, Italy (38°26'1008"N, 15°59'991"E). At least three individuals (>15 years old) per species were used for experiments. Species were selected on the basis of their hydraulic strategies, with specific reference to their different degrees of isohydric/anisohydric stomatal behavior (Nardini et al. 2014). This was quantified by comparing midday leaf water potential (see below) recorded in spring (May) with values recorded in summer (July) (Nardini et al. 2014). The seasonal water potential drop was  $\leq 1$  MPa in *Ceratonia siliqua* L., *Laurus nobilis* L., *Nerium oleander* L., *Pistacia lentiscus* L. and *Quercus ilex* L. (isohydric species), while it was  $\geq 2$  MPa in

Table 1. List of the species under study providing acronym (as used in the text and in Tables 2 and 3 and Figures 3–5), seasonal leaf water potential drop ( $\Delta\Psi$ , as derived by comparing midday leaf water potential values recorded in spring with values recorded in summer), and relative hydraulic strategy (for details, see the text).

Species	Acronym	$\Delta\Psi$ (MPa)	Hydraulic strategy
<i>Ceratonia siliqua</i> L.	Cs	0.7	Isohydic
<i>Laurus nobilis</i> L.	Ln	0.5	Isohydic
<i>Nerium oleander</i> L.	No	0.3	Isohydic
<i>Pistacia lentiscus</i> L.	Pl	1.0	Isohydic
<i>Quercus ilex</i> L.	Qi	1.0	Isohydic
<i>Eucalyptus camaldulensis</i> Dehnh.	Ec	2.1	Anisohydric
<i>Myrtus communis</i> L.	Mc	2.1	Anisohydric
<i>Olea europaea</i> L.	Oe	2.5	Anisohydric
<i>Olea oleaster</i> Hoffmanns. & Link	Oo	2.5	Anisohydric

*Eucalyptus camaldulensis* Dehnh., *Myrtus communis* L., *Olea europaea* L. and *Olea oleaster* Hoffmanns. & Link (anisohydric species, Table 1). During the whole experiment, trees were not irrigated and received only natural precipitation.

### Diurnal changes in plant water status and gas exchange

Leaf conductance to water vapor ( $g_L$ ), transpiration rate ( $E_L$ ), leaf water potential ( $\Psi_L$ ) and xylem water potential ( $\Psi_x$ ) were measured in July between 12:30 and 15:30 h (midday) and on the following day between 7:00 and 8:30 h (morning) using a steady-state porometer (LI 1600, LiCor Inc., Lincoln, NE, USA) and a pressure chamber (3005 Plant Water Status Console, Soil Moisture Equipment Corp., Santa Barbara, CA, USA). Midday  $\Psi_L$  measurements were performed also in May to estimate the seasonal leaf water potential drop and identify species-specific hydraulic strategies (see above). Stem xylem pressure ( $\Psi_x$ ) was estimated on leaves (or leafy shoots) maintained wrapped in cling film for at least 1 h before measurements.

### Diurnal changes in loss of stem hydraulic conductivity

At least seven stems per species were collected in July between 12:30 and 15:30 h (midday) and on the following day between 7:00 and 8:30 h (morning). Branches ~60–80-cm long were cut under water and transported to the laboratory within ~10 min with their cut basal end immersed in water. This length corresponded to at least 1.5 times the maximum vessel length for the study species as estimated with the air-injection technique at a pressure ( $P$ ) of 35 kPa (Cohen et al. 2003). Then, branches were progressively cut under water (at 10-cm intervals) from the base until a 15-cm long segment of the 1-year-old shoot was obtained, and this was connected to a hydraulic apparatus (Sperry et al. 1988, Lo Gullo and Salleo 1991) to estimate the loss of hydraulic conductivity (PLC). The sampling procedure likely assured progressive relaxation of native xylem tension, thus avoiding possible artifacts arising from direct cutting of samples under high tension (Wheeler et al. 2013, Torres-Ruiz et al. 2015), while also avoiding

long-term rehydration that might induce refilling of embolized conduits (Trifilò et al. 2014b, Venturas et al. 2015). Stem samples were perfused with a reference solution at a  $P$  of 8.0 kPa until the flow ( $F$ ) became stable, and their initial xylem-specific hydraulic conductivity ( $K_s$ ) was calculated as  $(F/P) \times (L/A_s)$  where  $L$  is the sample length and  $A_s$  is the xylem cross sectional area. The reference solution was a commercial mineral water containing several ions (0.51 mM  $\text{Ca}^{2+}$ , 0.07 mM  $\text{Mg}^{2+}$ , 0.08 mM  $\text{Na}^+$ , 0.03 mM  $\text{NO}_3^-$ , 0.96 mM  $\text{HCO}_3^-$ , 0.15 mM  $\text{SO}_4^{2-}$ , 0.01 mM  $\text{Fe}^{2+}$ ) and with  $[\text{K}^+]$  adjusted to a value of 20 mM by adding KCl to the mineral water (Nardini et al. 2007).

The stems were then flushed at  $P = 0.2$  MPa for 20 min to remove embolism and flow was re-measured at  $P = 8.0$  kPa to obtain  $K_{\text{max}}$ . The PLC was calculated as

$$\text{PLC} = \left[ 1 - \left( \frac{K_s}{K_{\text{max}}} \right) \right] \times 100$$

All hydraulic measurements were performed at a temperature of 20 °C. The difference between mean values of PLC as measured at midday ( $\text{PLC}_{\text{midday}}$ ) and on the next morning ( $\text{PLC}_{\text{morning}}$ ) was defined as 'PLC recovery', indicating the extent of diurnal changes in xylem embolism rates.

### Measuring species-specific vulnerability to xylem embolism

Vulnerability curves were measured for each study species using the bench dehydration technique, which is currently considered as a reference technique (Cochard et al. 2013). Branches were cut under water in the morning (between 7:00 and 8:00 h) and transported to the laboratory enclosed in a plastic bag and with their basal end immersed in water (see above). Branches were left rehydrating for 3 h. Then, branches were removed from water and left dehydrating in the laboratory for different time intervals (up to 18 h) to induce different xylem pressure values and measure the corresponding  $K_s$ . Sample preparation and hydraulic measurements were performed as described above.

Stem xylem pressure ( $\Psi_x$ ) was estimated on leaves (or leafy shoots) wrapped in cling film for at least 1 h to allow water potential equilibration. Data of xylem-specific hydraulic conductivity of stems were plotted versus the corresponding  $\Psi_x$  values and a sigmoidal function was fitted to derive  $P_{50}$  and  $P_{88}$  values (xylem pressure inducing 50 and 88% loss of hydraulic conductivity, respectively) for each species. Species-specific hydraulic safety margins were estimated as the difference between  $P_{50}$  or  $P_{88}$  and  $\Psi_x$  values recorded in planta at midday.

### Wood density and capacitance

Stem samples (4–8 per species) similar to those used for hydraulic measurements, were collected in the field, cut with shears in 2-cm-long segments and rehydrated overnight by immersion in distilled water. After rehydration, the fresh volume of samples (without bark) was estimated using the water displacement

method described by Hughes (2005). Wood density was calculated as dry weight/fresh volume ratio, where dry weight samples were obtained by oven-drying samples at 70 °C for 24 h (after wood capacitance measurements, see below). In order to estimate the species-specific wood capacitance ( $C_{\text{wood}}$ ), the same wood samples used for volume measurements were first weighed with a digital balance (mod. AE220, Sartorius, Göttingen, Germany; accuracy  $\pm 0.1$  mg) to measure their saturated weight (TW), and their water potential ( $\Psi$ ) was measured using a Dewpoint Hygrometer (WP4, Decagon Devices, Pullman, WA, USA, Whalley et al. 2013). Samples were then air-dehydrated on the bench until a decrease of  $\sim 3\text{--}7\%$  of their weight was recorded, and their water potential was re-measured. After  $\Psi$  reading, samples were immediately weighed to estimate their fresh weight (FW) and the corresponding water loss was calculated as: FW–TW. This procedure was repeated until  $\Psi$  reached values of about  $-2$  MPa.  $C_{\text{wood}}$  was estimated as the ratio between the wood water content changes over the corresponding variation of water potential, and normalized by fresh volume. The relationship between wood water content and water potential was strictly linear in the  $\Psi$  range analyzed ( $R^2 < 0.98$ ). It has to be noted that our aim was to quantify  $C_{\text{wood}}$  as a proxy of the amount of living

cells in the wood, and not to actually measure the capacitance in the range of water potential experienced by plants in the field.

### Data analysis

Data were analyzed with SigmaStat 2.0 (SPSS, Inc., Chicago, IL, USA). Differences were tested with Student's *t*-test. The significance of correlations was tested using the Pearson product-moment coefficient (*P*-value).

## Results

Table 2 reports stem and leaf water potential values, as well as  $g_L$  and  $E_L$  values recorded at midday and on the following morning in all species under study.  $\Psi_x$  and  $\Psi_L$  values were lower at midday with respect to values recorded in the morning in all the species studied. The highest (least negative) midday values of both  $\Psi_x$  and  $\Psi_L$  (about  $-1.2$  and  $-1.4$  MPa, respectively) were recorded in *N. oleander*, while the lowest values, averaging  $-3.7$  MPa ( $\Psi_x$ ) and  $-4$  MPa ( $\Psi_L$ ), were recorded in *O. oleaster*. Substantial inter-specific differences were also recorded in terms of maximum diurnal values of  $g_L$  and  $E_L$ . The highest midday  $g_L$  values ( $>300$  mmol m $^{-2}$  s $^{-1}$ ) were recorded in *E. camaldulensis* and

Table 2. Mean values  $\pm$  SD of xylem water potential ( $\Psi_x$ ), leaf water potential ( $\Psi_L$ ), leaf stomatal conductance to water vapor ( $g_L$ ) and transpiration rate ( $E_L$ ) as measured at midday and on the following morning in the species under study. Asterisks indicate, for each measured parameter and species, significant differences of midday versus morning mean values for Student's *t*-test (\* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ). Refer to Table 1 for species acronyms.

Species	$\Psi_x$ (MPa) $n = 5$	$\Psi_L$ (MPa) $n = 5$	$g_L$ (mmol m $^{-2}$ s $^{-1}$ ) $n = 8$	$E_L$ (mmol m $^{-2}$ s $^{-1}$ ) $n = 8$
Cs				
Midday	$-1.1 \pm 0.15^{**}$	$-1.8 \pm 0.09^{***}$	$260 \pm 23^{***}$	$5.2 \pm 0.5^{***}$
Morning	$-0.9 \pm 0.01^{**}$	$-1.3 \pm 0.05^{***}$	$70 \pm 13^{***}$	$1.2 \pm 0.3^{***}$
Ln				
Midday	$-1.3 \pm 0.15^{***}$	$-1.5 \pm 0.2^{***}$	$100 \pm 10^{***}$	$1.5 \pm 0.5^{***}$
Morning	$-0.8 \pm 0.09^{***}$	$-0.9 \pm 0.1^{***}$	$35.8 \pm 10^{***}$	$0.3 \pm 0.1^{***}$
No				
Midday	$-1.2 \pm 0.2^{***}$	$-1.4 \pm 0.08^{***}$	$311.5 \pm 78.5^{***}$	$5.3 \pm 1.5^{***}$
Morning	$-0.5 \pm 0.1^{***}$	$-0.7 \pm 0.1^{***}$	$75.1 \pm 15.1^{***}$	$0.9 \pm 0.2^{***}$
Pl				
Midday	$-1.5 \pm 0.2^*$	$-2.1 \pm 0.1^{**}$	$200 \pm 20^{***}$	$3.2 \pm 0.3^{***}$
Morning	$-1.1 \pm 0.2^*$	$-1.6 \pm 0.2^{**}$	$89.6 \pm 15.7^{***}$	$1.2 \pm 0.1^{***}$
Qi				
Midday	$-1.6 \pm 0.3^{***}$	$-1.9 \pm 0.2^{**}$	$135.7 \pm 23^{***}$	$3.0 \pm 0.6^{***}$
Morning	$-0.5 \pm 0.03^{***}$	$-1.1 \pm 0.5^{**}$	$96.6 \pm 14^{***}$	$1.0 \pm 0.2^{***}$
Ec				
Midday	$-1.5 \pm 0.05^{***}$	$-2.8 \pm 0.1^{***}$	$393 \pm 85^{***}$	$3.7 \pm 0.6^{***}$
Morning	$-0.9 \pm 0.02^{***}$	$-1.3 \pm 0.1^{***}$	$61 \pm 20^{***}$	$0.4 \pm 0.1^{***}$
Mc				
Midday	$-2.6 \pm 0.5^*$	$-2.7 \pm 0.4^*$	$70 \pm 4^{***}$	$0.5 \pm 0.05^{***}$
Morning	$-1.8 \pm 0.6^*$	$-1.9 \pm 0.5^*$	$40 \pm 7.5^{***}$	$0.3 \pm 0.09^{***}$
Oe				
Midday	$-2.3 \pm 0.2^*$	$-2.9 \pm 0.2^{***}$	$155 \pm 10^{***}$	$3.1 \pm 1^{***}$
Morning	$-1.9 \pm 0.1^*$	$-2.0 \pm 0.2^{***}$	$80 \pm 20^{***}$	$1.0 \pm 0.3^{***}$
Oo				
Midday	$-3.7 \pm 0.2^{**}$	$-3.9 \pm 0.1^{**}$	$115 \pm 15^{***}$	$1.3 \pm 0.07^{***}$
Morning	$-2.9 \pm 0.4^{**}$	$-2.9 \pm 0.7^{**}$	$53 \pm 7^{***}$	$0.6 \pm 0.09^{***}$



*N. oleander*, while the lowest ( $<100 \text{ mmol m}^{-2} \text{ s}^{-1}$ ) were recorded in *L. nobilis* and *M. communis*. *Ceratonia siliqua* and *N. oleander* showed  $E_L$  values at midday higher than  $\sim 5 \text{ mmol m}^{-2} \text{ s}^{-1}$  while  $E_L < 1.5 \text{ mmol m}^{-2} \text{ s}^{-1}$  was recorded in *L. nobilis*, *M. communis* and *O. oleaster*.

Despite daily changes of xylem water potential, not all species showed significant diurnal changes in PLC (Figure 1). In particular, *P. lentiscus*, *Q. ilex*, *M. communis* and *O. oleaster* experienced PLC of  $\sim 40\text{--}50\%$  both at midday and on the following morning. In contrast, the other species showed daily changes of PLC in the order of  $15\text{--}25\%$  (PLC recovery, see also Table 3) when comparing midday versus morning values. Diurnal changes in embolism rate were similar in the isohydric and anisohydric species. In fact, the mean value of the diurnal PLC recovery as recorded in the five isohydric specimens under study was statistically similar to the value of the four species with an anisohydric behavior (i.e.,  $11.5 \pm 4.2$  vs  $6.6 \pm 3.8$ , respectively,

$P$ -value = 0.427). It can be noted, moreover, that no statistically significant correlation was found when species-specific diurnal PLC changes were plotted versus the corresponding diurnal drop in leaf water potential ( $\Psi_{\text{midday}} - \Psi_{\text{morning}}$ ) ( $R^2 = 0.22$ ,  $P$ -value = 0.2). Moreover, no correlation was observed between species-specific maximum vessel length (see Table 3) and maximum diurnal PLC ( $R^2 = 0.04$ ,  $P$ -value = 0.58) or diurnal PLC changes ( $R^2 = 0.15$ ,  $P$ -value = 0.29).

Xylem vulnerability to embolism formation varied widely across species (Figure 2, Table 3). *Nerium oleander* was the most vulnerable species with  $P_{50}$  and  $P_{88}$  values around  $-1.5$  and  $-2.8$  MPa, respectively. Higher resistance to embolism was recorded for *C. siliqua*, *L. nobilis*, *Q. ilex*, *M. communis* and *O. europaea*, where  $P_{50}$  ranged between  $-2.1$  and  $-3.3$  MPa. The most negative  $P_{50}$  values (less than  $-4$  MPa) were recorded in *P. lentiscus*, *E. camaldulensis* and *O. oleaster*. In these species, the  $P_{88}$  values were also remarkably negative (less than  $-7$  MPa). Safety margins calculated against

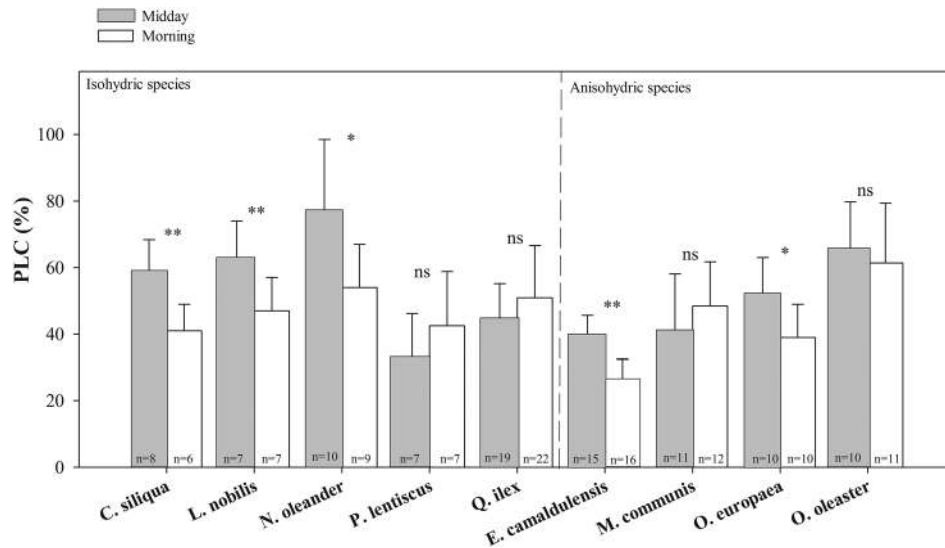


Figure 1. Mean values  $\pm$  SD of PLC as measured at midday (gray columns) and on the following morning (white columns) in the species under study. Asterisks indicate, for each species, significant differences of midday versus morning values for Student's  $t$ -test. \* $P < 0.05$ ; \*\* $P < 0.01$ ; ns, not significant.

Table 3. Values of xylem water potential inducing 50% ( $P_{50}$ ) and 88% ( $P_{88}$ ) loss of hydraulic conductivity (PLC),  $P_{50}$  and  $P_{88}$  safety margin (estimated as the difference between  $P_{50}$  or  $P_{88}$  and  $\Psi_L$  values recorded in planta at midday, respectively), and PLC recovery (estimated as the difference between the PLC measured at midday and on the following morning). Mean values  $\pm$  SD of WD, wood capacitance ( $C_{\text{wood}}$ ) and maximum vessel length ( $L_{\text{max}}$ ) as measured in all species under study are also reported. Refer to Table 1 for species acronyms.

Species	$P_{50}$ (MPa)	$P_{88}$ (MPa)	$P_{50}$ safety margin (MPa)	$P_{88}$ safety margin (MPa)	PLC recovery	WD ( $\text{g cm}^{-3}$ )	$C_{\text{wood}}$ ( $\text{kg MPa}^{-1} \text{ m}^{-3}$ )	$L_{\text{max}}$ (m)
Cs	-2.6	-2.9	1.5	1.8	17.4	$0.5 \pm 0.07$	$16.5 \pm 1.8$	$0.38 \pm 0.05$
Ln	-2.5	-4.8	1.2	3.5	16	$0.4 \pm 0.03$	$39.3 \pm 9.8$	$0.40 \pm 0.04$
No	-1.5	-2.8	0.3	1.5	22.5	$0.3 \pm 0.08$	$102.5 \pm 5.9$	$0.33 \pm 0.03$
Pl	-4.1	-7.0	2.6	5.5	0	$0.5 \pm 0.04$	$31.1 \pm 6.8$	$0.19 \pm 0.03$
Qi	-3.3	-5.7	1.7	4.1	0	$0.6 \pm 0.03$	$37.2 \pm 8.2$	$0.45 \pm 0.01$
Ec	-4.6	Less than -7.5	3.1	/	13.3	$0.5 \pm 0.03$	$33.8 \pm 1.9$	$0.52 \pm 0.06$
Mc	-3.1	-5.2	0.5	2.6	0	$0.6 \pm 0.02$	$15.2 \pm 3.9$	$0.16 \pm 0.02$
Oe	-2.1	-4.8	-0.2	2.5	13	$0.7 \pm 0.02$	$27.4 \pm 7.8$	$0.40 \pm 0.03$
Oo	-4.1	Less than -7	0.4	/	0	$0.8 \pm 0.01$	$13.6 \pm 1.1$	$0.37 \pm 0.03$

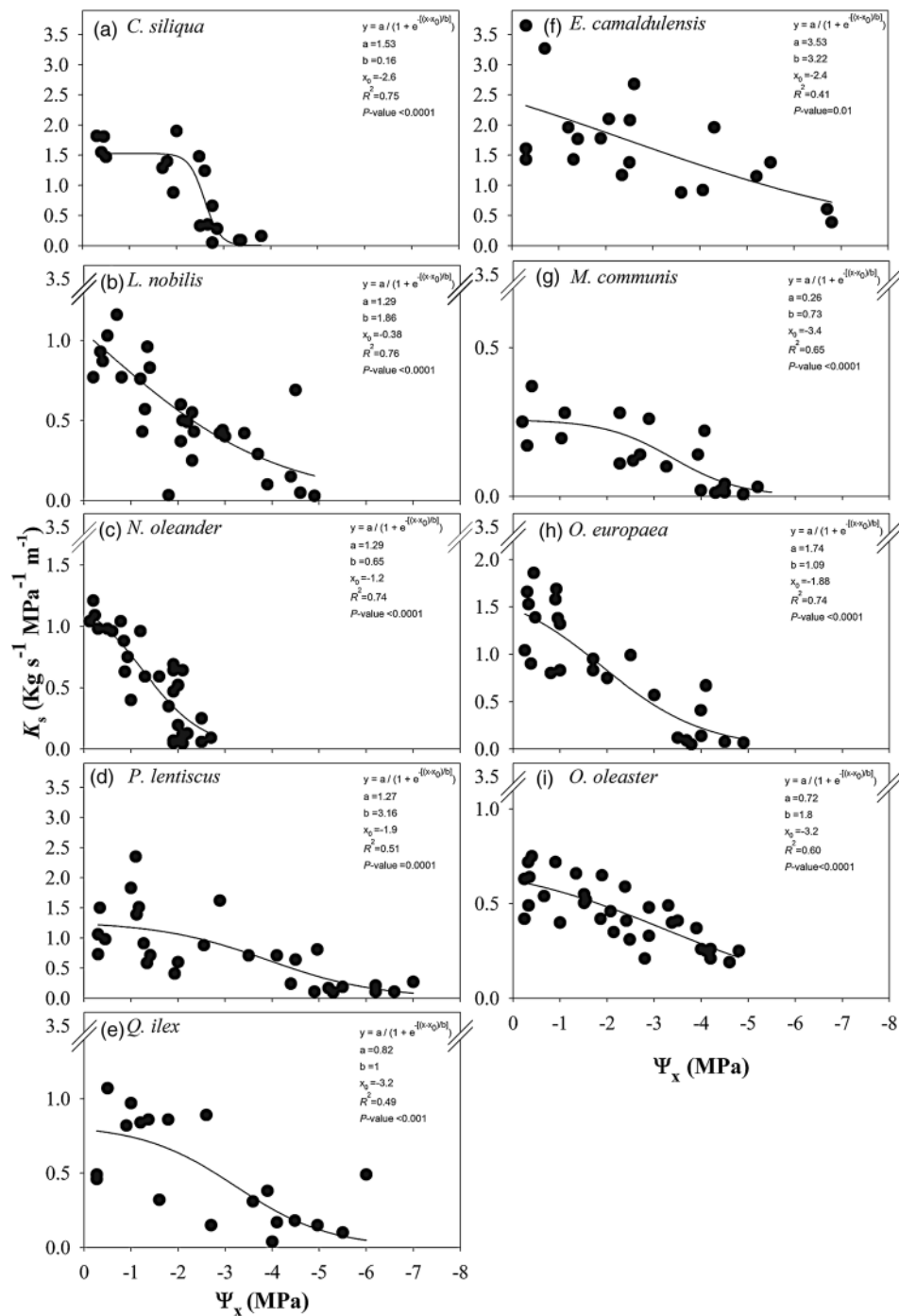


Figure 2. Xylem-specific hydraulic conductivity of stems ( $K_s$ ) versus xylem pressure ( $\Psi_x$ ) of isohydric (a–e) and anisohydric (f–i) species. Regression equation, coefficient values, correlation coefficients ( $R^2$ ) and  $P$ -values are also reported.

$P_{50}$  ranged between  $-0.2$  MPa (in *O. europaea*) and  $3.1$  MPa (as recorded in *E. camaldulensis*) while the same parameter calculated against  $P_{88}$  varied from  $1.5$  MPa (in *N. oleander*) to  $5.5$  MPa (in *P. lentiscus*).

*Olea europaea* and *O. oleaster* showed the highest WD ( $\sim 0.7$  and  $0.8$  g cm $^{-3}$ , respectively) while *N. oleander* had the lowest one (i.e.,  $\sim 0.3$  g cm $^{-3}$ ).  $C_{wood}$  values were  $\sim 15$  kg MPa $^{-1}$  m $^{-3}$  in *C. siliqua*, *M. communis* and *O. oleaster*,  $\sim 35$  kg MPa $^{-1}$  m $^{-3}$  in

*L. nobilis*, *P. lentiscus*, *Q. ilex*, *E. camaldulensis* and *O. europaea*, and as high as  $103$  kg MPa $^{-1}$  m $^{-3}$  in *N. oleander*.

When diurnal PLC changes were plotted versus  $P_{50}$  and  $P_{88}$  values, a significant correlation ( $P$ -value  $< 0.05$ ) was recorded (Figure 3). However, the correlation was stronger when diurnal PLC changes were plotted versus  $P_{88}$  values than versus  $P_{50}$  values (i.e.,  $R^2 = 0.69$  versus  $R^2 = 0.51$ , respectively). Moreover, when diurnal PLC changes were plotted versus safety margin

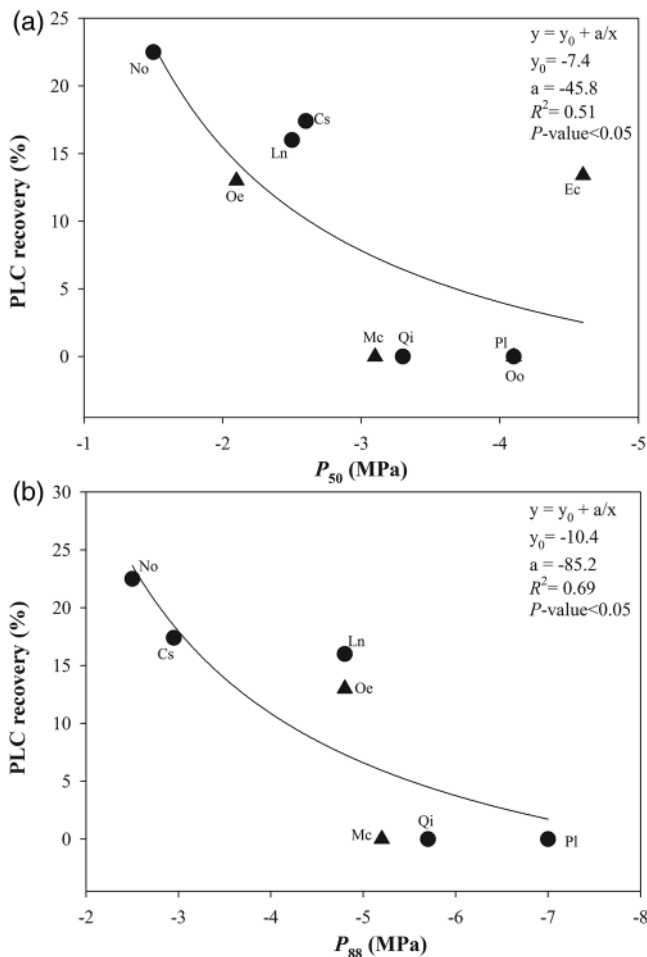


Figure 3. Values of PLC recovery as a function of: (a) xylem water potential inducing 50% loss of hydraulic conductivity ( $P_{50}$ ) and (b) xylem water potential inducing 88% loss of hydraulic conductivity ( $P_{88}$ ) across isohydric (circles) and anisohydric (triangles) species. Species acronyms are reported in Table 1. Regression equation, coefficient values, correlation coefficients ( $R^2$ ) and  $P$ -values are also reported.

toward  $P_{50}$ , a linear correlation was observed only for isohydric species (Figure 4a). In contrast, a significant correlation (according to an exponential decay equation,  $P$ -value  $< 0.05$ ) was recorded between the diurnal PLC changes and the safety margin toward  $P_{88}$  for all the study species, regardless of their hydraulic strategy (Figure 4b).

Wood density was linearly correlated across all species with minimum diurnal leaf water potential, diurnal PLC changes and wood capacitance (Figure 5a, d and e). In contrast, significant relationships between  $P_{50}$ ,  $P_{50}$  safety margin values and WD were recorded only for the isohydric species (Figure 5b and c).

## Discussion

Our data reveal possible functional links between diurnal changes in embolism level, hydraulic strategies and WD/capacitance across nine dry forest trees. Species more vulnerable to xylem embolism apparently operate with relatively narrow safety

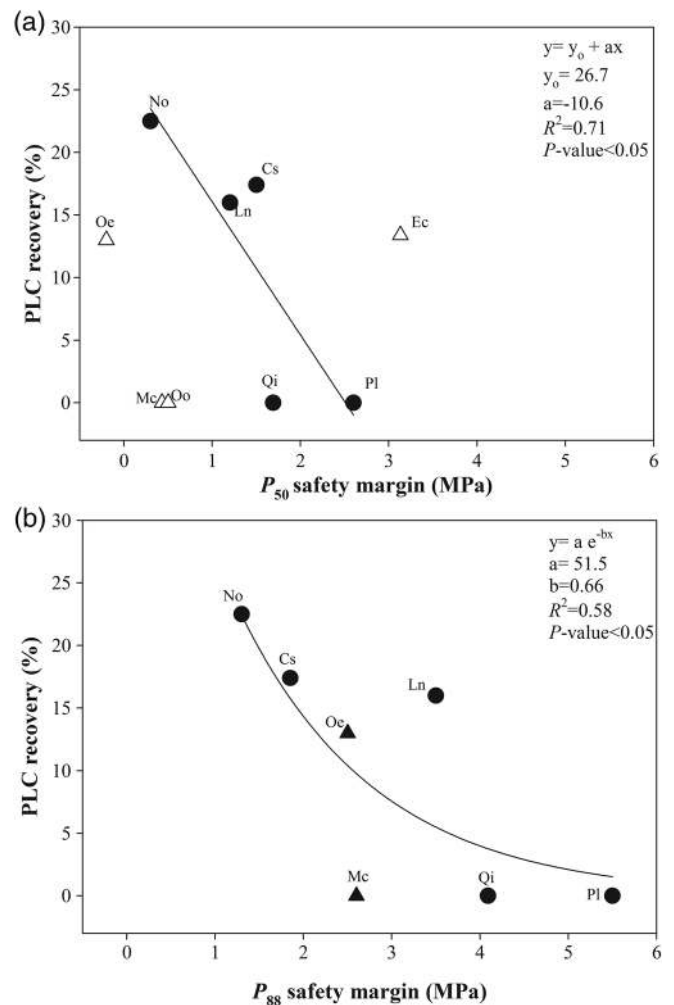


Figure 4. Values of PLC recovery plotted as a function of: (a)  $P_{50}$  safety margin and (b)  $P_{88}$  safety margin values across isohydric (circles) and anisohydric (triangles) species. Species acronyms are reported in Table 1. Regression equation, coefficient values, correlation coefficients ( $R^2$ ) and  $P$ -values are also reported. Data for the species represented by open triangles were not used to fit the regression line.

margins against catastrophic and irreversible hydraulic failure ( $P_{88}$ ) and hence experience large losses of hydraulic conductance on a daily basis. However, these species also display significant nocturnal PLC recovery, apparently favored by low WD and high xylem capacitance.

Diurnal changes in embolism level were recorded in *C. siliqua*, *L. nobilis*, *N. oleander*, *E. camaldulensis* and *O. europaea*, but not in *P. lentiscus*, *Q. ilex*, *M. communis* and *O. oleaster* (Figure 1, Table 3). Interestingly, all species had minimum PLC values of ~30–40% even in the early morning, and in some species (i.e., *L. nobilis*, *N. oleander*, *Q. ilex*, *O. oleaster*) PLC never dropped below 50% (Figure 1). Values of PLC measured in planta were higher than those predicted from vulnerability curves on the basis of stem water potentials (Figures 1 and 2). This discrepancy can be explained by native embolism that had likely been removed in samples utilized for vulnerability curves (i.e., branches cut under

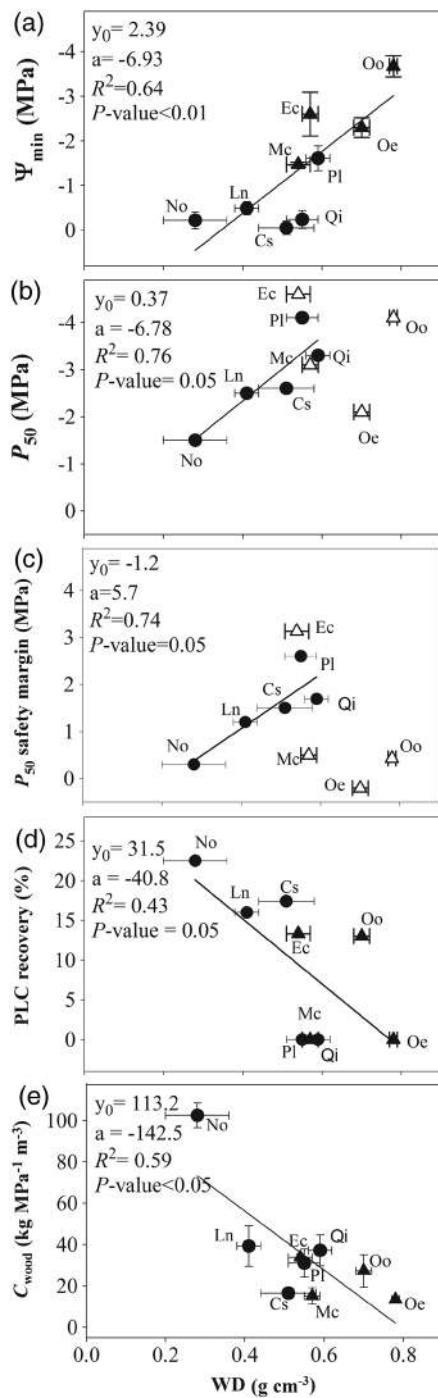


Figure 5. Values of: (a) leaf water potential as measured at midday ( $\Psi_{\min}$ ), (b) xylem water potential inducing 50% loss of hydraulic conductance wood capacitance ( $P_{50}$ ), (c)  $P_{50}$  safety margin, (d) PLC recovery and (e) wood capacitance ( $C_{\text{wood}}$ ) as a function of WD across isohydric (circles) and anisohydric (triangles) species. White symbols refer to species excluded by the regression. Species acronyms are reported in Table 1. Regression curves are expressed by the following function:  $y = y_0 + ax$ . Values of coefficients  $y_0$  and  $a$  as well as of correlation coefficients ( $R^2$ ) and  $P$ -values are reported.

water and maintained enclosed in a plastic bag, with their basal end immersed in water for 3 h) but not in those used for midday PLC estimation. In other words, it is possible that this high native

PLC was due to the presence of irreversibly embolized conduits that could not be repaired over night-time in planta, and indeed previous studies on Mediterranean species reported similarly high embolism rates in plants apparently not severely water-stressed (Tognetti et al. 1998, Nardini and Pitt 1999, Nardini et al. 2000, Torres-Ruiz et al. 2013). Nevertheless, leaf conductance to water vapor was higher than  $\sim 70 \text{ mmol m}^{-2} \text{ s}^{-1}$  in all study species (Table 2), suggesting that embolism levels recorded were still compatible with maintenance of adequate leaf water supply and gas exchange (Trifilò et al. 2014a).

Diurnal changes in stem xylem embolism have been reported by previous studies (Zwieniecki and Holbrook 1998, Christman et al. 2012, Trifilò et al. 2014a) and interpreted as evidence for the occurrence of diurnal cycles of embolism formation/repair (Nardini et al. 2011). This view has been recently challenged by experiments reporting induction of artifactual embolism in stem cuts from plants while xylem conduits are under substantial tension, even if the cut is made under water (Wheeler et al. 2013). On this basis, diurnal changes in embolism would simply arise because stems sampled at midday or in the morning are cut while under different native xylem tension, and this would introduce different levels of artifactual embolism at different day times. In order to avoid the artifact, Wheeler et al. (2013) suggested fully rehydrating stem samples before hydraulic measurements, in order to relax native tension. Although partial support of this phenomenon has been provided by direct micro-CT observations (Torres-Ruiz et al. 2015), the so-called 'Wheeler effect' has not been confirmed in two independent studies (Scoffoni and Sack 2015, Venturas et al. 2015), or has been attributed to artifactual refilling of previously embolized conduits during the rehydration/relaxation procedure (Trifilò et al. 2014b, Venturas et al. 2015). Taking into account that the 'Wheeler effect' would arise from cutting stems under different xylem tension at different day times, we should expect to see a relationship across species between the magnitude of diurnal PLC changes and the corresponding diurnal changes in xylem pressure. In other words, apparent nocturnal embolism recovery should be a function of the difference between xylem water potential measured at midday versus early morning. This was apparently not the case in the present study, as diurnal PLC changes recorded in nine woody species were not correlated to the corresponding diurnal drop in plant water potential ( $\Psi_{\text{midday}} - \Psi_{\text{morning}}$ ). Moreover, the 'Wheeler effect' should be expected to be larger in species with longer vessels, but in this study we did not find any correlation between species-specific vessel lengths and related changes in PLC. These findings, coupled with the sampling procedure that assured partial relaxation of xylem pressures from field sampling to laboratory hydraulic measurements (see Materials and methods), suggests that diurnal changes in stem hydraulic conductivity recorded in the study species accurately reflected patterns of embolism formation during diurnal water stress and conduits' refilling during night-time. Nocturnal recovery of PLC occurred at



xylem pressures higher than  $-1.0$  MPa, with the only exception of *O. europaea* where a moderate ( $<15\%$ ) but significant recovery of PLC apparently occurred at  $\Psi_x$  as low as  $-2.0$  MPa. These data are in accordance with previous studies. As an example, Nardini et al. (2008) reported refilling of sunflower leaf xylem at a tension of  $-0.3$  MPa, a value consistent with observations by Christman et al. (2012) documenting nocturnal refilling in oak at sap tension averaging  $-0.5$  MPa. Brodersen et al. (2010) showed that embolism reversal was initiated in grapevine stems under tensions between  $-1.6$  and  $-0.7$  MPa, and similar results were obtained by Zufferey et al. (2011). Salleo et al. (1996) and Tyree et al. (1999) reported partial embolism recovery in laurel at xylem pressures around  $-1.0$  MPa, a value consistent with those allowing xylem refilling in spruce (Mayr et al. 2014). In the present study, diurnal changes in PLC were related to species-specific  $P_{50}$  and  $P_{88}$  values, thus possibly suggesting that species characterized by high resistance to embolism formation did not experience xylem pressures low enough to induce substantial embolism formation, or they were not able to reverse embolism as efficiently as more vulnerable species. More interestingly, the magnitude of embolism recovery was clearly correlated with safety margins against  $P_{88}$  across all study species (i.e., both isohydric and anisohydric) (Figure 4). In contrast, when diurnal embolism recovery was plotted versus safety margins as calculated toward  $P_{50}$ , a linear correlation was recorded only for isohydric species (Figure 4a). Our data suggest that safety margins calculated against  $P_{50}$  cannot be always considered as reliable and general indicators of the species-specific risk of hydraulic failure, while reinforcing the hypothesis that PLC  $\sim 88\%$  represents a limit that when approached likely requires plants to be able to refill their embolized conduits in order to survive upon drought stress relief (i.e., Tyree et al. 2002, Urli et al. 2013).

Our data also suggest that daily changes in PLC are not intrinsically different in species characterized by an isohydric strategy versus those more markedly anisohydric (Figure 1). Nevertheless, it must be noted that a large spectrum of variance exists within the binary classification of isohydric and anisohydric species (Franks et al. 2007, West et al. 2012, Klein 2014), and it cannot be excluded that the number of species tested in our study was too low to capture significant variance and correlations in the above traits. Therefore, our present results must be taken with caution.

In our study,  $P_{50}$  was correlated to WD only across isohydric species, while the correlation was weak when all species were included in the regression (Figure 5). This would suggest that high mechanical reinforcement of the xylem does not invariably translate into high resistance to xylem embolism (Hoffmann et al. 2011, Markesteijn et al. 2011), implying that caution should be exercised in employing structural proxies of plant resistance to drought stress in ecological models. Interestingly, both diurnal  $\Psi_{\min}$  and diurnal PLC changes were significantly correlated to WD, which in turn had a significant impact on wood

hydraulic capacitance (Figure 5), as previously reported in different assemblages of woody species (Domec et al. 2006, Pratt et al. 2007, Scholz et al. 2007, Meinzer et al. 2008, Richards et al. 2014). In previous studies, high wood capacitance has been interpreted as adaptive in terms of potential water release under water stress, a mechanism that would act as a dynamic component of the hydraulic safety of plant water transport systems (Meinzer et al. 2009, Gleason et al. 2014, Richards et al. 2014). More specifically, it has been hypothesized that low WD and high capacitance would allow plants to buffer the diurnal and/or seasonal drought-induced drop in xylem pressure, thus avoiding embolism formation. Our data do not support this view, as the largest diurnal changes in PLC were recorded in the species with lower WD and higher capacitance, suggesting that high wood capacitance did not help plants to prevent substantial embolism build-up over the day. On the contrary, our data suggest a prominent role of high wood capacitance in driving nocturnal embolism recovery in some drought-adapted trees. High wood capacitance has been shown to be partially driven by a higher fraction of living cells in wood (Scholz et al. 2007), with special reference to axial parenchyma (Zheng and Martinez-Cabrera 2013). There is current consensus around the crucial role played by vessel-associated living cells in embolism reversal, mainly as a source of osmotica necessary to generate positive pressures driving water influx into embolized conduits (Salleo et al. 2004, 2009, Secchi and Zwieniecki 2012), and possibly as direct sources of water needed to refill the conduits (Brodersen et al. 2010). A possible role of high wood capacitance in favoring embolism reversal has been previously suggested (Johnson et al. 2012, Meinzer and McCulloh 2013, Ogasa et al. 2013), but to the best of our knowledge our study is the first reporting tight correlations between these traits.

Our data suggest that future research efforts should be aimed at quantifying relationships between wood anatomical/functional traits, plant metabolism and drought resistance/resilience of trees. In fact, there is growing concern about the impact of global change-type droughts on tree mortality (McDowell et al. 2013, Bussotti et al. 2015), and information about key traits underlying species-specific resistance/resilience to such events is urgently needed. Drought-induced tree mortality is a complex event and current hypotheses suggest that hydraulic failure and carbon starvation play prominent roles (McDowell et al. 2011, Sevanto et al. 2014). Our data support the hypothesis of close functional links between water and carbon metabolism, with special reference to the maintenance of integrity of the water transport system (Zwieniecki and Secchi 2014). In fact, embolism recovery was largest in species with high wood capacitance, which presumably underlies a relationship with the amount of living cells in the wood. Vessel-associated cells have been shown to store significant amounts of starch (Bucci et al. 2003, Salleo et al. 2004) that is apparently degraded upon embolism induction to provide soluble sugars and generate osmotic

pressures driving conduits refilling (Salleo et al. 2009, Secchi and Zwieniecki 2012). Plants analyzed in the present study were subjected to a 'normal' summer drought and, in fact, stomatal aperture was apparently not severely constrained at the time of measurements. Hence, it is very likely that our plants were not experiencing any significant carbon starvation, and nocturnal refilling was possible in species with high wood capacitance and presumably high levels of non-structural carbohydrates (NSC) near embolized vessels. However, long-term drought might limit plant carbon gain, leading to depletion of NSC reserves and impairment of embolism repair and plant water status recovery upon stress relief. To the best of our knowledge, there are no studies available in the literature specifically addressing the relationships between NSC concentration and embolism recovery, and our present data suggest that this information is critical for a deep understanding of tree performance under extreme drought. Further, our data indicate that relationships between NSC and embolism formation/recovery are more likely to occur in species more vulnerable to xylem embolism, operating with narrow safety margins referenced to  $P_{88}$ , and characterized by high wood capacitance and low WD.

In conclusion, our results show that embolism formation under drought stress cannot be simply predicted on the basis of species-specific  $P_{50}$  or  $P_{88}$  values. Diurnal xylem embolism formation is more likely in species operating with narrow safety margins, but these species are also characterized by wood traits apparently favoring embolism reversal during night-time, even at substantial residual xylem tension. While safety margins can be adequately considered as 'hydraulic proxies' for species-specific vulnerability to drought (Delzon and Cochard 2014), the 'metabolic proxies' for species-specific resilience to drought remain to be quantified, and studies investigating relationships between wood capacitance, NSC concentration and embolism reversal are called for to provide answers to urgent questions about forest vulnerability to climate changes.

### Conflict of interest

None declared.

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