Coping with drought-induced xylem cavitation: coordination of embolism repair and ionic effects in three Mediterranean evergreens

Patrizia Trifilò¹,4, Piera M. Barbera², Fabio Raimondo¹, Andrea Nardini³ and Maria A. Lo Gullo¹

¹Dipartimento di Scienze Biologiche e Ambientali, Università di Messina, salita F. Stagno D’Alcontres 31, 98166 Messina, Italy; ²Dipartimento di Agraria, Università Mediterranea di Reggio Calabria, Feo di Vito, 89122 Reggio Calabria, Italy; ³Dipartimento di Scienze della Vita, Università di Trieste, Via L. Giorgieri 10, 34127 Trieste, Italy; ⁴Corresponding author (ptrifillo@unime.it)

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Embolism repair and ionic effects on xylem hydraulic conductance have been documented in different tree species. However, the diurnal and seasonal patterns of both phenomena and their actual role in plants’ responses to drought-induced xylem cavitation have not been thoroughly investigated. This study provides experimental evidence of the ability of three Mediterranean species to maintain hydraulic function under drought stress by coordinating the refilling of xylem conduits and ion-mediated enhancement of stem hydraulic conductance (Kstem). Vessel grouping indices and starch content in vessel-associated parenchyma cells were quantified to verify eventual correlations with ionic effects and refilling, respectively. Experiments were performed on stems of Ceratonia siliqua L., Olea europaea L. and Laurus nobilis L. Seasonal, ion-mediated changes in Kstem (ΔKstem) and diurnal and/or seasonal embolism repair were recorded for all three species, although with different temporal patterns. Field measurements of leaf specific stem hydraulic conductivity showed that it remained quite constant during the year, despite changes in the levels of embolism. Starch content in vessel-associated parenchyma cells changed on diurnal and seasonal scales in L. nobilis and O. europaea but not in C. siliqua. Values of ΔKstem were significantly correlated with vessel multiple fraction values (the ratio of grouped vessels to total number of vessels). Our data suggest that the regulation of xylem water transport in Mediterranean plants relies on a close integration between xylem refilling and ionic effects. These functional traits apparently play important roles in plants’ responses to drought-induced xylem cavitation.

Keywords: drought stress, gas exchange, potassium, refilling, starch-to-sugar conversion, xylem anatomy.

Introduction

Long-distance water transport in vascular plants occurs through a highly efficient conduit network, the xylem. According to the cohesion–tension theory, water is transported in the xylem under tension, i.e., in a metastable state (Tyree and Zimmermann 2002). Under such conditions, water is prone to cavitation, resulting in xylem embolism (Nardini et al. 2011b). Xylem embolism is a relatively common consequence of drought and freezing stress, and it can be detected even in well-watered plants (Choat et al. 2012). When embolism occurs, the unavoidable reduction of xylem hydraulic conductance (Kstem) leads to partial or total stomatal closure and reduction of photosynthetic rates (Brodribb 2009). This is especially exacerbated during prolonged and very intense drought events (Brodribb and Holbrook 2006, McDowell 2011).

Plants are able to cope with embolism-induced loss of hydraulic conductance through (i) production of new xylem (e.g., Ameglio et al. 2002), (ii) repair of embolized xylem conduits (novel refilling, Salleo et al. 1996), and (iii) ion-mediated
enhancement of residual stem hydraulic conductance ($K_{stem}$, the so-called ‘ionic effect’, Zwieniecki et al. 2001). While the production of new xylem conduits requires relatively long time intervals, refilling and ionic effects are potentially effective mechanisms for the short-term regulation of xylem water transport and, as a consequence, they could help plants face short-term drought stress.

Embolism reversal has been documented in >30 plant species (Brodersen and McElrone 2013) and, according to the most recent findings (Secchi and Zwieniecki 2012), the process is apparently based on an osmotic mechanism. Briefly, the currently accepted hypothesis proposes that the driving force to refill with water gas-filled conduits is generated by the input of solutes into the residual sap in the embolized vessel. The most reasonable osmotica involved are sugars such as sucrose obtained from starch depolymerization in xylem parenchyma cells and exported to conduits (e.g., Bucci et al. 2003, Salleo et al. 2006, Regier et al. 2009, Secchi and Zwieniecki 2011). However, the involvement of proteins and/or inorganic ions such as K+ cannot be excluded (Tyree et al. 1999, Neumann et al. 2010). Moreover, it is not clear whether phloem, whose integrity is apparently required for refilling to occur (Bucci et al. 2003, Salleo et al. 2004), is involved in the unloading of sugars and water into refilling conduits and/or in exportation of chemical signals for starch depolymerization (Nardini et al. 2011a).

The occurrence of an ion-mediated regulation of $K_{stem}$ has also been documented in several angiosperm species (Nardini et al. 2011b). The ionic effect is supposed to be a consequence of the polyelectrolytic nature of pit membrane components (Zwieniecki et al. 2001, Gortan et al. 2011, Dusotoit-Coucaud et al. 2013). Recently, Lee et al. (2012) have suggested that the thickness, rather than porosity as originally hypothesized, of the bordered pit membrane changes in response to sap ion concentrations. From this view, ion-induced reduction of pit membrane thickness would be responsible for the decrease of hydraulic resistance of the pit water pathway. However, the exact mechanism underlying the ionic effect is still controversial (Van Doorn et al. 2011, Santiago et al. 2013). Positive correlations between anatomical features of pits and vessels, such as vessel grouping, and the ionic effect are in general agreement with the involvement of pit apertures (Jansen et al. 2011), but several questions are still unresolved. Most importantly, it is still unclear whether embolism repair and ionic effects are effectively adopted and coordinated by plants to alleviate xylem dysfunction under field conditions, although some recent studies have reported the occurrence of both phenomena in field-grown plants (Trifilò et al. 2003, 2008, 2011, Salleo et al. 2009).

On the basis of the above, it is reasonable to suppose that the ability of plants to recover from, or to compensate for, cavitation could improve their competitiveness and resistance to drought stress, especially in Mediterranean-type biomes where climate imposes high selective pressure for adequate leaf water supply (Nardini et al. 2014). However, clear-cut evidence supporting this hypothesis is still largely lacking. More information about the regulation of xylem water transport in Mediterranean plants is needed in order to predict the impact of increased summer drought frequency/intensity on vegetation in this biome (Martínez-Vilalta et al. 2002, Giorgi and Lionello 2008, Matusik et al. 2013). Hydraulic failure (i.e., plant desiccation due to inadequate water transport after cavitation) has been invoked as a possible causal factor of tree dieoff during drought events (McDowell et al. 2008, Hoffmann et al. 2011, Nardini et al. 2013). Therefore, a deeper understanding of species-specific regulation of xylem water transport could allow clarification of species-specific responses to aridity and prediction of responses of forest ecosystems to climate changes (Choat et al. 2012, Nardini et al. 2013, Sack and Scoffoni 2013).

This paper describes the diurnal and seasonal occurrence of embolism repair and ionic effects in three Mediterranean species (Ceratonia siliqua L., Olea europaea L. and Laurus nobilis L.) growing in the same habitat, with the aim of determining whether these mechanisms are employed by plants during periods of drought stress. Both laboratory and field measurements were performed, and the possible correlations between seasonal changes in ionic effects and xylem anatomical features as well as between embolism repair and starch availability were investigated.

**Materials and methods**

Experiments were performed in January (winter), May (spring), July (summer) and October (autumn) 2012 on 1-year (during January) to 2-year (in October) old stems collected from at least two adult specimens of three evergreen tree species growing on the campus of the Department of Biological Sciences and Environment, University of Messina, Italy (38° 15′ 41.34″ N; 15° 35′ 51.67″ E). The species investigated were C. siliqua L. (carob), O. europaea L. (olive) and L. nobilis L. (laurel). Carob and olive are typical components of dry sclerophyllous Mediterranean woodlands (Pignatti 1982), while L. nobilis is a sclerophyllous evergreen more restricted to mesic sites. Measurements were carried out at two different times of day: between 7:00 and 8:30 h (morning) on branches with low transpiration rates (<0.8 mmol m⁻² s⁻¹) and between 12:30 and 15:30 h (midday) on sun-exposed branches with high transpiration rates (see Table 2). During the entire experiment, trees were not irrigated and received only natural precipitation. Prewax leaf water potential ($\Psi_{Pw}$) under these conditions was measured on three basal covered leaves per species per season using a pressure chamber (3005 Plant Water Status Console, Soil Moisture Equipment Corp., Santa Barbara, CA, USA) (Table 1).

**Measuring gas exchange and leaf water potential**

Leaf conductance to water vapour ($g_{w}$), transpiration rate ($E_{L}$) and leaf water potential ($\Psi_{s}$) were measured on a diurnal/
seasonal scale on at least eight leaves per species using a portable steady-state porometer (LI 1600, LiCor Inc., Lincoln, NE, USA) and the pressure chamber (see above). Air temperature ($T_{air}$), air relative humidity (RH) and photosynthetically active radiation (PAR) were also recorded using the sensors of the porometer cuvette.

**Measuring xylem sap potassium concentration**

In order to estimate diurnal and seasonal changes in xylem sap potassium concentration ([K$^+$]), 80- to 120-cm-long shoots were collected, immediately defoliated and transported to the laboratory. Xylem sap was extracted using the vacuum chamber technique (Améglio et al. 2004). The cut surface of the shoot was rinsed with deionized water to remove phloem sap residues and debris and put into a 1.5-ml Eppendorf vial, which rested in a beaker filled with ice to minimize evaporation during sap collection. Potassium concentration was then measured with a [K$^+$]-selective electrode (Cardy Compact Ion Meter, Model C-131, Horiba Ltd, Kyoto, Japan).

**Measuring ion-mediated changes in hydraulic conductance**

This experiment was designed to assess the magnitude of the ionic effect and its eventual changes on a seasonal basis. Ion-mediated increases in stem hydraulic conductance ($\Delta K_{stem}$) were tested in fully hydrated samples by perfusing samples with KCl solutions at increasing concentrations (10, 25 and 50 mM). The $K_{stem}$ measured under KCl perfusion was compared with values recorded under perfusion with a reference solution (commercial mineral water containing 0.51 mM Ca$^{2+}$, 0.07 mM Mg$^{2+}$, 0.08 mM Na$^+$, 0.03 mM NO$_3^-$, 0.96 mM HCO$_3^-$, 0.15 mM SO$_4^{2-}$ and 0.01 mM F$^-$, with [K$^+$] adjusted to 3 mM). This reference perfusion fluid was selected to prevent spurious hydraulic effects caused by the use of deionized water (Nardini et al. 2007). Stem samples were cut off under distilled water and immediately transported to the laboratory where they were re-cut to a length of 18 cm and connected to a hydraulic apparatus (Xyl'Em, Xylem Embolism Meter, Bronkhorst, Montigny les Cormeilles, France). This stem length was selected on the basis of the species-specific vessel length (see below), in order to minimize the number of conduits cut open at both ends (Gascó et al. 2006). Samples were first flushed with the reference solution at $P = 0.2$ MPa for 20 min to remove native embolism. The $K_{stem}$ was recorded at $P = 7.5$ kPa with reference fluid and then with KCl solutions at increasing concentrations. At each concentration level, measurements were continued until the flow became stable (~10 and 30 min for the reference and KCl solutions, respectively). At least five stems per species and per month were tested.

In order to evaluate the actual occurrence of ion-mediated $K_{stem}$ increase in planta, as well as its impact on plant water transport efficiency, measurements of $\Delta K_{stem}$ were performed on samples collected in the field both in the morning and at midday on a seasonal scale. $\Delta K_{stem}$ of samples with native embolism levels were thus obtained comparing $K_{stem}$ measured with a perfused solution with [K$^+$] equal to the concentration recorded in planta (at the same time of day and month in which samples were collected) with values measured by perfusing the reference solution. Measurements were performed on the same samples used to measure the loss of stem hydraulic conductance (see below).

It should be noted that data gathered on fully hydrated samples represent a potential increase of $K_{stem}$ in response to potassium ions. By contrast, data gathered on samples with different degrees of native embolism provide information about the actual enhancement of $K_{stem}$ associated with the ionic effect in planta, taking into account both the degree of xylem dysfunction (see Gascó et al. 2006) and xylem sap [K$^+$].

**Measuring percentage loss of stem hydraulic conductance**

On the basis of (i) the different [K$^+$] recorded at midday versus morning and (ii) the seasonal changes in the magnitude of the ionic effect (see the Results section), an experiment was designed to estimate the actual buffering effect of the ion-mediated enhancement of residual $K_{stem}$ on the overall loss of stem hydraulic conductance, on both a diurnal and a seasonal scale. Daily and seasonal changes of ‘reference’ percentage loss of stem hydraulic conductance (PLC) values were also estimated with the reference solution in order to check the ability of stems to refill embolized xylem conduits.

At least eight stems per species per month were collected between 12:30 and 15:30 h (midday) and on the following day between 7:00 and 8:30 h (morning). Stems ~50 cm long were excised in the field under distilled water. Samples were rapidly transported to the laboratory with the cut proximal end immersed in water. About 10–20 min after sampling, a segment ~18 cm long was cut from the distal portion of the sample. The time interval from initial sampling to preparation of the sample for hydraulic measurements was probably sufficient to partially relax xylem tension (Wheeler et al. 2013). Samples were connected to the Xyl'Em and native $K_{stem}$ ($K_s$) was immediately measured using a solution (see above) with [K$^+$] adjusted to mimic concentrations recorded at the same time of day and month (see the Results section). Then, $K_{stem}$ was re-measured at low pressure (i.e., 7.5 kPa) with the reference solution at low potassium concentration (3 mM [K$^+$], $K_{stem}$ [K$^+$]). Stems were then flushed with the reference solution at a pressure of 0.2 MPa for 20 min to remove embolism and samples were re-measured at low pressure with the reference solution with low potassium ($K_{max}$) or with [K$^+$] adjusted to concentrations recorded in planta, thus obtaining native $K_{max}$ ($K_{max,n}$). This procedure allowed us to calculate the PLC at 3 mM [K$^+$] and the PLC at native [K$^+$] (PLC$_n$) as

\[
(1 - K_{stem}/K_{max}) \times 100 \quad \text{and} \quad (1 - K_{stem}/K_{max,n}) \times 100, \nonumber
\]

respectively.
In summary, PLC at 3 mM [K+] estimated the theoretical impact of embolism when not buffered by ionic effects (because the ion concentration of the reference solution was very low), thus providing information about the eventual occurrence of embolism reversal on a diurnal scale. By contrast, PLCi, provided an estimate of the actual loss of water transport capacity suffered by plants taking into account ion-mediated compensation mechanisms (Trifilò et al. 2011).

All hydraulic measurements were performed at a temperature of 20 °C.

**Xylem anatomy**

To select the length of stem samples to be used for hydraulic measurements (see above), the maximum xylem conduit lengths of four stems per species were determined using the silicone-injection technique (Sperry et al. 2005). Samples were connected to the hydraulic apparatus, flushed with water at $P = 0.2$ MPa to remove native embolism and then injected at $P = 0.5$ MPa for 3 h with silicone (Rhodorsil RTV-141, Rhodia, Cranbury, NJ, USA) mixed with a blue pigment (Pentasol, Prochima, Pesaro, Italy). After ~12 h (i.e., the time needed for silicone hardening) stems were progressively cut into 2-cm-long segments. Conduits filled with blue silicone and the total number of conduits were counted under a microscope on sequential cross-sections, and the conduit length distribution was calculated as proposed by Sperry et al. (2005). The length of stems containing at least 60% of intact conduits was 14 cm for carob and olive and 12 cm for laurel.

The stems used for PLC measurements were cross-sectioned for anatomical analysis. Sections 30 µm thick were obtained with a microtome and briefly rinsed with distilled water before immersion for 1 min in a Lugol solution (iodine–potassium iodide) (Salleo et al. 2004, 2009). After rinsing samples again to remove excess stain, sections were observed under a microscope (Laborlux S, Leitz Esselte, Leitz GmbH, Stuttgart, Germany) equipped with a digital camera (Leica DC300F, Leica Camera AG, Solms, Germany) connected to a computer. For each section, eight to 15 different microscopic fields, together covering at least 60% of the section, were observed at ×25 magnification. The solitary vessel index, $V_s$ (ratio of total number of solitary vessels to total vessel groupings), and the vessel multiple fraction, $F_{vm}$ (ratio of grouped vessels to total number of vessels), were calculated (Jansen et al. 2011). Moreover, the percentage of xylem parenchyma cells with ‘high starch content’ (HSC-cells, i.e., starch granules filled >50% of the cell lumen) in relation to the total number of xylem parenchyma cells per cross-section was recorded (Salleo et al. 2004, 2009). This procedure allowed us to check whether seasonal changes of ionic effect and refilling were related to vessel grouping and starch content of xylem parenchyma cells, respectively.

**Measurements of leaf specific stem hydraulic conductivity in planta**

In order to get additional experimental support for laboratory measurements of $K_{stem}$, leaf specific stem hydraulic conductivity ($LSC_{stem}$) was estimated in the field, both in the morning and at midday, using the evaporative flux method (EFM) (Nardini et al. 2010, Trifilò et al. 2013).

On the evening preceding the experiments, two apical and two basal leaves were selected from 80–120-cm-long branches and wrapped in plastic film and aluminium foil to equilibrate $\Psi_y$ with that of the adjacent stem xylem ($\Psi_{x, apical}$ and $\Psi_{x, basal}$, respectively). $LSC_{stem}$ was calculated as

$$LSC_{stem} = \frac{E_i}{(\Psi_{x, basal} - \Psi_{x, apical})}$$

where $E_i$ was measured on a leaf inserted ~70–110 cm distally to those selected for estimating $\Psi_{x, basal}$ and near the leaf later used for estimating $\Psi_{x, apical}$, while $i$ was the distance between the basal and apical leaves. All $LSC_{stem}$ values were corrected to a temperature of 20 °C to account for changes in water viscosity. Measurements were performed on at least six morning and six midday shoots.

It has to be noted that $LSC_{stem}$ is not equivalent to values of $K_{stem}$ because in the first the transpiration rate per unit leaf area of a single leaf was measured, while the values of stem hydraulic conductance take into account the actual and total flow through the stem xylem. However, estimating $LSC_{stem}$ in planta could give useful information about the actual impact of both refilling and ionic effect mechanisms on maintaining hydraulic function. In any case, data recorded with EFM must be interpreted with caution because of the intrinsic experimental limits of this methodology, i.e., the possible inaccurate estimates of branch-level transpiration rate and related water potential drop. For example, the boundary layer conductance in the chamber of the porometer was probably different from that experienced by bagged leaves used to estimate water potential gradient.

**Statistics**

Data were analysed with the SigmaStat 2.0 (SPSS, Inc., Chicago, IL, USA) statistics package. Differences on seasonal and daily scales in measured parameters and between treatments were tested for each species using one-way ANOVA and Tukey’s tests.

**Results**

**Environmental and water relations data**

During measurements, the PAR was ~40 µmol m$^{-2}$ s$^{-1}$ in the morning, and ranged between 1000 (January) and 1600 µmol m$^{-2}$ s$^{-1}$ (July) at midday (Table 1). Air humidity was higher in the morning whereas $T_{air}$ reached maximum values (~30 °C) during July at midday.
Table 2 shows the $g_L$, $E_L$ and $Ψ_L$ values recorded in the morning and midday in the three study species. In *C. siliqua*, $g_L$ ranged between 60 and 250 mmol m$^{-2}$ s$^{-1}$ in the morning and at midday, respectively. The most negative $Ψ_L$ values were recorded in July at midday (about $-1.73$ MPa), while the morning values were higher and not different throughout the year (about $-0.8$ MPa). In olive, $g_L$ was always $-70$ mmol m$^{-2}$ s$^{-1}$ in the morning versus $-200$ mmol m$^{-2}$ s$^{-1}$ during January and October at midday. In laurel, $g_L$ of the morning samples was $-45$ mmol m$^{-2}$ s$^{-1}$ throughout the year. At midday, $g_L$ was $-140$ mmol m$^{-2}$ s$^{-1}$ in January, May and October, while a statistically significant decrease was recorded in July ($-100$ mmol m$^{-2}$ s$^{-1}$). In these two species, the lowest $Ψ_L$ values were recorded in July at midday ($-2.9 ± 0.36$ MPa in olive and $-1.87 ± 0.17$ MPa in laurel), despite the occurrence of partial stomatal closure.

In all the three species, the transpiration rates as well as the pressure drops between stem base and apex ($Ψ_{basal} - Ψ_{apical}$) were larger at midday than in the early morning.

**Xylem sap [K]$^+$**

Xylem sap [K]$^+$ changed on a diurnal and seasonal time scale in all the species studied (Figure 1). In carob, the early morning values of xylem sap [K]$^+$ ranged from $-2$ mM (in January) to $-5$ mM (in the other months). At midday, [K]$^+$ increased to $-5$ mM in January and $12$ mM in October. In olive and laurel, the values of xylem sap [K]$^+$ were quite constant throughout the year in the morning hours ($-5$ mM), while midday [K]$^+$ increased to $14$ mM.

**Measurements of ion-mediated changes in $K_{stem}$**

Fully hydrated samples of carob and olive trees showed the highest $ΔK_{stem}$ values when perfused with 25 mM KCl solution, while *L. nobilis* showed a further increase of $K_{stem}$ to 50 mM KCl (data not shown). Hence, Figure 2 reports maximum $ΔK_{stem}$ values obtained on fully hydrated samples by perfusing solutions enriched with 25 mM KCl for carob and olive and 50 mM KCl for laurel. The magnitude of the $ΔK_{stem}$ differed in the three species, as in *C. siliqua* it was less than +10% in January, about +20% in May and July, and reached the maximum in October (about +34%) (Figure 2a). In *O. europaea*, the ionic effect was lowest in May (10%) and quite constant during the other months (about +30%) (Figure 2b). In *L. nobilis*, $ΔK_{stem}$ increased by 30% in January, but was significantly less in July and October (about +20%) while no ionic effect was recorded in May (Figure 2c).

Different ion-mediated increases in stem hydraulic conductance were recorded in embolized samples when midday samples were compared with morning ones, using potassium concentrations mimicking values recorded in planta (Figure 3). In all the species, $ΔK_{stem}$ in the morning was always very low or zero, as a consequence of the low native [K]$^+$. By contrast, $ΔK_{stem}$ increased significantly at midday in all seasons except in January for carob and in May for olive and laurel. It can be noted that $ΔK_{stem}$ of embolized samples at midday showed, on a seasonal basis, a trend quite similar to that recorded in fully hydrated stems, although with different and generally higher values. In particular, $ΔK_{stem}$ in carob was about +5% in January, +35% in May and July, and +80% in October (Figure 3a). In olive, values of about +50% were recorded in all study periods, except in May when $ΔK_{stem}$ was only about +18% (Figure 3b). In laurel, $ΔK_{stem}$ was as high as +45% in all months except in May (Figure 3c). It is worth noting that increases of $K_{stem}$ to $-80$% (in carob) and $-45$–50% (in olive and laurel) were recorded in embolized samples by perfusing solution with only 15 mM KCl, corresponding to native potassium levels in the xylem sap. By contrast, values not much higher than about +35% were recorded in the same months in fully hydrated samples perfusing 25 mM (for carob and olive) or 50 mM (for laurel) KCl solutions (Figures 2 and 3).

**Daily and seasonal PLC changes**

The PLC of *C. siliqua* during perfusion with the reference solution did not differ between morning and midday in January (PLC = 25%) (Figure 4a). By contrast, in May and July PLC values as high as 55 and 40% were recorded in midday and morning samples, respectively. In October, an increase of PLC was observed both at midday and in the morning (80 and
Table 2. Means ± SD (n = 8) of $g_s$, $E_t$, $Ψ_t$ and pressure drops between the stem base and apex ($Ψ_{basal} - Ψ_{apical}$) of morning and midday leaves of *C. siliqua*, *O. europaea* and *L. nobilis* recorded in January, May, July and October 2012.

<table>
<thead>
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<th></th>
<th>January Morning</th>
<th>Midday</th>
<th>May Morning</th>
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<th>July Morning</th>
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<th>October Morning</th>
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<tr>
<td>$g_s$ (mmol m$^{-2}$ s$^{-1}$)</td>
<td>65 ± 9a</td>
<td>245 ± 24b</td>
<td>59 ± 8a</td>
<td>251 ± 74b</td>
<td>83 ± 7a</td>
<td>275 ± 75b</td>
<td>57 ± 7a</td>
<td>250 ± 70b</td>
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<td>$E_t$ (mmol m$^{-2}$ s$^{-1}$)</td>
<td>0.8 ± 0.3a</td>
<td>1.9 ± 0.3b</td>
<td>0.6 ± 0.2a</td>
<td>3.9 ± 1.2c</td>
<td>0.8 ± 0.2a</td>
<td>4.9 ± 1.5c</td>
<td>0.7 ± 0.2a</td>
<td>4 ± 1c</td>
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<td>$Ψ_t$ (MPa)</td>
<td>−0.78 ± 0.04</td>
<td>−99 ± 0.09b</td>
<td>−0.72 ± 0.09</td>
<td>−1.23 ± 0.12c</td>
<td>−0.77 ± 0.1a</td>
<td>−1.73 ± 0.18d</td>
<td>−0.85 ± 0.06a</td>
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<td>($Ψ_{basal} - Ψ_{apical}$) (MPa)</td>
<td>0.04 ± 0.01a</td>
<td>0.08 ± 0.02b</td>
<td>0.04 ± 0.01a</td>
<td>0.13 ± 0.03c</td>
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<td>$g_s$ (mmol m$^{-2}$ s$^{-1}$)</td>
<td>70 ± 7a</td>
<td>213 ± 40b</td>
<td>61 ± 7a</td>
<td>148 ± 44c</td>
<td>78 ± 10a</td>
<td>128 ± 23c</td>
<td>76 ± 8a</td>
<td>204 ± 54b</td>
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<td>$E_t$ (mmol m$^{-2}$ s$^{-1}$)</td>
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<td>0.7 ± 0.1a</td>
<td>4.1 ± 1.3c</td>
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<td>$Ψ_t$ (MPa)</td>
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<td>−1.22 ± 0.09ad</td>
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<td>($Ψ_{basal} - Ψ_{apical}$) (MPa)</td>
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<td>0.24 ± 0.04d</td>
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<tr>
<td>$g_s$ (mmol m$^{-2}$ s$^{-1}$)</td>
<td>48 ± 10a</td>
<td>158 ± 32b</td>
<td>41 ± 12a</td>
<td>138 ± 22b</td>
<td>50 ± 15a</td>
<td>100 ± 18c</td>
<td>47.3 ± 11a</td>
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<td>0.65 ± 0.2a</td>
<td>3.99 ± 1.4c</td>
<td>0.4 ± 0.2a</td>
<td>3.0 ± 0.8c</td>
</tr>
<tr>
<td>$Ψ_t$ (MPa)</td>
<td>−0.5 ± 0.06a</td>
<td>−1.03 ± 0.07b</td>
<td>−0.49 ± 0.1a</td>
<td>−1.58 ± 0.4c</td>
<td>−1.43 ± 0.5c</td>
<td>−1.87 ± 0.17d</td>
<td>−0.92 ± 0.3b</td>
<td>−1.9 ± 0.2d</td>
</tr>
<tr>
<td>($Ψ_{basal} - Ψ_{apical}$) (MPa)</td>
<td>0.06 ± 0.01a</td>
<td>0.1 ± 0.03bc</td>
<td>0.04 ± 0.01a</td>
<td>0.12 ± 0.04b</td>
<td>0.08 ± 0.02ac</td>
<td>0.22 ± 0.02d</td>
<td>0.05 ± 0.02a</td>
<td>0.2 ± 0.02d</td>
</tr>
</tbody>
</table>

Different letters indicate significant differences for Tukey's pairwise comparisons ($P < 0.001$).

Figure 1. Mean values ± SD (n = 8) of xylem sap $[K^+]$ (mM) as measured in (a) *C. siliqua* (black columns), (b) *O. europaea* (white columns) and (c) *L. nobilis* in 2012. Different letters indicate significant differences for Tukey’s pairwise comparisons ($P < 0.001$).
Embolism repair and ionic effects in Mediterranean trees

C. siliqua, and the midday loss of hydraulic conductance was further buffered by the increase of $[\text{K}^+]$ in the xylem sap that enhanced residual $K_{\text{stem}}$.

In O. europaea, diurnal embolism repair was observed in January, in July and, to a lesser extent, in October (Figure 4b).

The PLC at 3 mM $[\text{K}^+]$ in midday branches was $\sim$55% in January, July and October, while in the morning it declined to $\sim$50% in January and $\sim$40% in July and October. When midday branches were perfused with the reference solution mimicking the sap $[\text{K}^+]$ recorded in planta, the PLC was significantly lower in all study periods except May. In laurel, diurnal changes of standard PLC were observed in all the 4 months (Figure 4c). Ion-mediated changes in stem hydraulic conductance allowed lower midday PLC in January, July and October, but not in May.

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**Figure 2.** Means ± SD ($n=5$) of the percentage increase of stem hydraulic conductance ($\Delta K_{\text{stem}}$) of fully hydrated samples as induced by 25 mM KCl in carob (a) and olive (b) or 50 mM KCl in laurel (c) compared with the reference solution. Different letters indicate significant differences for Tukey’s pairwise comparisons ($P<0.001$).

**Figure 3.** Means ± SD ($n=8$) of the percentage $\Delta K_{\text{stem}}$ of embolized samples as induced by a reference solution enriched with $[\text{K}^+]$ similar to the concentration recorded in planta compared with the reference solution. Different letters indicate significant differences for Tukey’s pairwise comparisons ($P<0.001$). Grey and dashed grey columns refer to midday and morning samples, respectively.
Xylem anatomy

The starch content in stem parenchyma cells changed during the season in all three species (Table 3). In *C. siliqua*, the maximum percentage of HSC cells was observed in May (~80%) and the minimum in July and October (~4%). In each study period, however, morning and midday HSC percentages were not different, except in January. In olive, the percentage of HSC changed on a diurnal scale in January and July, when it was...
higher in the morning than at midday. A similar diurnal difference in the amount of HSC was observed in laurel in all the study periods with the only exception of January, when no changes in starch content were detected.

All species showed a significantly higher value of the \( V_s \) in May (Table 4), when it was 0.57 ± 0.07 (C. siliqua), 0.56 ± 0.04 (O. europaea) and 0.49 ± 0.06 (L. nobilis). A different behaviour was recorded for \( F_{VM} \) of the three species. Vessel multiple fraction of carob was highest in July and October (~0.30). In olive, \( F_{VM} \) peaked in January (0.35 ± 0.03), while laurel showed the highest values in January, July and October (~0.30).

When midday values of \( \Delta K_{stem} \) were plotted versus corresponding PLC at 3 mM [K\(^+\)] as recorded in all species on a seasonal basis, a positive correlation emerged between the two parameters (Figure 5a). Moreover, \( \Delta K_{stem} \) was significantly correlated to \( F_{VM} \) (Figure 5b). Therefore, ion-mediated changes of stem hydraulic conductance increased as a function of both higher xylem dysfunction and higher vessel grouping. No correlation was found between the diurnal change in stem starch content (i.e., HSC of midday branches) and the daily change of PLC (data not shown).

**Field measurements of LSC\(_{stem} \)**

Figure 6 reports values of LSC\(_{stem} \) as measured in planta using the EFM technique. In all the three species, no statistically significant differences were recorded in morning versus midday samples throughout the year. In C. siliqua, values of LSC\(_{stem} \) of ~20 mmol m\(^{-1} \) s\(^{-1} \) MPa\(^{-1} \) were recorded except in the early morning samples in July and October when LSC\(_{stem} \) was <12 mmol m\(^{-1} \) s\(^{-1} \) MPa\(^{-1} \) (Figure 6a). In olive and laurel samples, LSC\(_{stem} \) ranged from ~5 mmol m\(^{-1} \) s\(^{-1} \) MPa\(^{-1} \) to ~12 mmol m\(^{-1} \) s\(^{-1} \) MPa\(^{-1} \) throughout the year (Figure 6b and c).

**Discussion**

The main goal of the present study was to assess whether Mediterranean plants adopt ion-mediated regulation of xylem water transport as well as embolism repair processes to cope with seasonal drought stress. Our data suggest that the drought responses of the species studied were indeed based on the fine regulation of long-distance water transport, obtained by both diurnal and seasonal xylem refilling patterns, as well as by transient ion-mediated enhancement of \( K_{stem} \).

**Ionic effect**

Changes in the magnitude of the ionic effect as well as in xylem sap [K\(^+\)] were recorded in all three species on a seasonal scale (Figures 1–3). As a consequence of the increase of [K\(^+\)] on a diurnal basis (Figure 1), \( K_{stem} \) was significantly enhanced at midday, especially in the months in which the xylem sensitivity to ions was higher (October for carob, January, June and October for olive, and January for laurel). As a consequence, native PLC measured with solutions mimicking the actual sap potassium content was found to be substantially different from PLC standard values recorded with a reference
low-[K⁺] solution (Figure 4). In particular, when the ionic effect was present and [K⁺] was high, the PLC was lower than the reference PLC. Nevertheless, these data and the higher ΔKstem values recorded in embolized stems than in fully hydrated samples (Figures 2 and 3) indicate that the magnitude of ion-mediated enhancement of Kstem is itself a function of PLC, as already reported in previous studies (Gascó et al. 2006, Trifilò et al. 2008). The positive correlation recorded between ΔKstem and PLC values (Figure 5a) clearly confirms this view and suggests that the ionic effect is an effective mechanism utilized by plants to alleviate the impact of xylem embolism, as previously hypothesized but only partially demonstrated in planta (Gascó et al. 2007, Trifilò et al. 2008, 2011). Our data also suggest that drought-stressed plants enrich their xylem sap with potassium ions to enhance the magnitude of the ionic effect, especially when embolism-induced xylem dysfunction develops in their transport systems. In fact, the highest sap [K⁺] (Figure 1) was recorded in the months in which the ionic effect was greater and PLC was larger (Figures 2 and 4). In any case, the possible source of ions loaded into xylem sap remains unclear although the most likely candidate is the phloem (Zwieniecki et al. 2004, Metzner et al. 2010).
Data reported in the present study reinforce the notion that the relative magnitude of the ionic effect is related to vessel arrangement (Jansen et al. 2011), as both $\Delta K_{stem}$ and sap ionic strength were found to be higher in the species/season when xylem conduits appeared highly grouped (Figure 5). Jansen et al. (2011) showed a highly significant correlation between the ionic effect and some vessel grouping parameters, especially the portion of vessel walls in contact with neighbouring vessels. The ionic effect is larger in xylem with highly grouped vessels because the fraction of intervessel contacts and related radial flows through pit membranes is correlated to vessel grouping. We did not quantify the anatomical and biochemical features of pit membranes, but our results reinforce the hypothesis that xylem anatomy, and in particular the extent of vessel-to-vessel connections, is directly involved in determining the ionic effect, at both inter- and intra-specific (i.e., seasonal) scales. Recent and classical studies have suggested that cambial activity and vessel arrangement in early versus late wood may be affected by different environmental events (e.g., fire, drought, salt stress, flooding and low temperature) (e.g., Madany et al. 1982, Lovisolo and Schubert 1998, St George et al. 2002, Schmitz et al. 2006, Ellemann et al. 2009, Trifilò et al. 2013). This apparently confers to plants a large flexibility in terms of xylem construction in response to micro- and macro-environmental changes (Fonti et al. 2010), thus making it possible to modulate water transport as a function of environmental factors (Nardini et al. 2012, Zwieniecki and Secchi 2012). Of course, this does not exclude the hypothesis that ion-mediated changes of $K_{stem}$ can be modulated rapidly also via modifications of the biochemical features of pit membrane structure (Zwieniecki and Secchi 2012).

Refilling

All species under study showed the ability to reverse xylem embolism (Figure 4). In fact, when midday losses of stem hydraulic conductance were compared with morning PLC values as recorded with the same reference solution (i.e., PLC at 3 mM [K+]), changes in the amount of embolism became apparent in all three species. In particular, in carob trees the diurnal xylem refilling was evident in all months except January. In olive and laurel, refilling was not observed only in May (Figure 4b and c).

Taking into account that the xylem vessel diameter of all three species ranged between 15 and 80 $\mu$m (data not shown), and on the basis of Henry’s law, it can be predicted that in the species under study a passive bubble collapse could have occurred in a range of pressure between $-0.02$ and $-0.0018$ MPa. Therefore, a passive refilling mechanism, especially in some months, could not be ruled out considering unusual high (i.e., not very negative) values of $\Psi_{PD}$.

Previous studies have led to the hypothesis that refilling processes are driven by the depolymerization of starch (Bucci et al. 2003, Salleo et al. 2006, Secchi et al. 2011). This hypothesis is only partially confirmed by our data (Table 3 and Figure 4). In olive and laurel, a correlation between HSC and refilling was apparent, and no diurnal change of stem starch content was observed when xylem repair was absent (i.e., in May and October for olive and in January for laurel). However, in carob, the starch content of HSC was always low except in May. Moreover, in this species HSC did not change diurnally, despite the occurrence of recovery of embolized conduits. There are four possible explanations for these findings: (i) starch is not involved in the process of refilling, at least in some species; (ii) polysaccharides other than starch are involved in refilling in carob; (iii) starch deposits are much bigger than the amount of sugars actually required to generate the osmotic pressures sufficient to refill embolized conduits; or (iv) the method used to estimate the changes of starch content is only semi-quantitative and does not permit exact quantification of starch amount, so that it cannot be excluded that small changes of starch content occurred also in carob wood parenchyma cells.

Secchi et al. (2011) have reported that upon embolism formation, the metabolic pathways for the transcription of monosaccharide are down-regulated, while those for disaccharides and starch are up-regulated. Nevertheless, the metabolic coordination between carbon supply, growth and storage in trees is still largely unknown, and carbon supply (via photosynthesis) and overall demand (including probably the refilling process) are often not synchronized (Sala et al. 2012).

Based on our present results, we can suggest that the presence of starch in xylem parenchyma cells does not necessarily imply an ability to repair embolized conduits. However, starch seems to be a pre requisite for refilling, at least in some species like O. europaea and L. nobilis.

Functional links between ionic effects and embolism repair

The mechanisms of embolism repair and ion-mediated compensation of $K_{stem}$ loss were apparently activated by the three species on different temporal scales to optimize the water transport from soil to sites of photosynthesis even under moderate drought stress conditions. In accordance, LSC$_{stem}$ values, as measured in planta with the EFM, remained always constant throughout the study period, despite substantial accumulation of embolism during some months (Figure 6). In fact, despite (i) the rainfall in Messina during the summer of 2012 being higher than the mean recorded from 1971 to 2000 (120.6 mm versus 58.3 mm; source: Italian Air Force Meteorological Service) and, as a consequence, (ii) the lowest $\Psi_{PD}$ values being higher than $-0.1$ MPa in all three species (Table 1), the dynamic water stress developing during the daytime was sufficient to significantly impair xylem function (Figure 4). However, despite standard values of PLC >60–70% being recorded in
some months in our species, the actual PLC estimated by perfusing solutions mimicking native [K+] (i.e., loss of $K_{stem}$ in planta) was never higher than ~50%.

According to data reported in the literature, the $\Psi_L$ at the turgor loss point between May and September is about $-2.1$ MPa in *C. siliqua*, $-3.1$ MPa in *O. europaea* and $-2.6$ MPa in *L. nobilis* (Lo Gullo and Salleo 1988, Correia et al. 2001). In *C. siliqua*, midday $\Psi_L$ was constantly higher than $-1.7$ MPa even in the driest period, and no reduction of gas exchange was observed (Table 2). In olive, the most negative values of $\Psi_L$ (about $-2.9$ MPa) were recorded in July. Such $\Psi_L$ values are common for olive even under moderate water stress (e.g., Guerfel et al. 2009, Quero et al. 2011). Nevertheless, also in this species, the turgor loss point was never reached, and $g_L$ was reduced by only ~30%. Moreover, in laurel the minimum $\Psi_L$ measured was about $-1.87$ MPa, (i.e., still above zero turgor) and gas exchange rates decreased by ~30%. In other words, data reported in the present study suggest that PLC values of ~50–60%, although suggesting substantial xylem dysfunction, were still compatible with maintenance of adequate leaf water supply and relatively high gas exchange rates.

Previous studies have suggested ionic effects and embolism repair as useful mechanisms to face drought and salt stress and to optimize the delivery and use of resources such as light and nutrients (Trifilò et al. 2008, 2011, 2013, Nardini et al. 2010). However, the present study is the first in which the regulation of xylem water transport is shown to depend on a strongly coordinated integration of both mechanisms on daily and seasonal time scales. This reinforces the idea that an efficient transport system must be able to modulate water transport as a function of micro-environmental changes (Nardini et al. 2012, Zwieniecki and Secchi 2012). This physiological trait could probably play an important role in shaping vegetation composition, distribution and plant survival in response to extreme climatic events. According to our current understanding, global climate changes are the cause of widespread forest decline (e.g., Allen et al. 2010, McDowell 2011, Choat et al. 2012). The impact of extreme droughts on vegetation is predicted to be higher in fragile biomes such as Mediterranean-type ecosystems (Martínez-Vilalta et al. 2002, Matusik et al. 2013). In turn, a differential impact of severe drought events on vegetation could be expected on local scales as a function of the species-specific ability to regulate $K_{stem}$ in response to the risk of hydraulic failure. Understanding different species’ ability in xylem transport modulation could be useful in predicting the impact of drought events on vegetation. However, more research is needed to clarify the occurrence of both refilling and ionic effect mechanisms in larger plant species data sets, as well as the eventual correlations between vulnerability to cavitation, xylem transport regulation and safety margins and, last but not least, the possible variability in the ability to regulate water transport at different drought stress levels.

**Conflict of interest**

None declared.

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**References**


Gortan E, Nardini A, Salleo S, Jansen S (2011) Pit membrane chemistry influences the magnitude of ion-mediated enhancement of xylem...
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