Chemical inhibition of xylem cellular activity impedes the removal of drought-induced embolisms in poplar stems – new insights from micro-CT analysis

Francesca Secchi[1] (iD), Chiara Pagliarani[2] (iD), Silvia Cavalletto[1] (iD), Francesco Petruzzellis[3] (iD), Giulia Tonel[1] (iD), Tadeja Savi[4] (iD), Giuliana Tromba[5] (iD), Maria Margherita Obertino[1] (iD), Claudio Lovisolo[1] (iD), Andrea Nardini[3] (iD) and Maciej A. Zwieniecki[6] (iD)

[1]Department of Agricultural, Forest and Food Sciences, University of Turin, Largo Paolo Braccini 2, Grugliasco 10095, Italy; [2]Institute for Sustainable Plant Protection, National Research Council, Strada delle Cacce 73, Torino 10135, Italy; [3]Dipartimento di Scienze della Vita, University of Trieste, via Giorgieri 10, Trieste 34127, Italy; [4]Institute of Botany, Department of Integrative Biology and Biodiversity Research, BOKU, Gregor-Mendel-Straße 33, Vienna 1180, Austria; [5]Elettra-Sincrotrone Trieste, Area Science Park, Basovizza, Trieste 34149, Italy; [6]Department of Plant Sciences, University of California Davis, One Shields Avenue, Davis, CA 95616, USA

Summary

- In drought-stressed plants a coordinated cascade of chemical and transcriptional adjustments occurs at the same time as embolism formation. While these processes do not affect embolism formation during stress, they may prime stems for recovery during rehydration by modifying apoplast pH and increasing sugar concentration in the xylem sap.

- Here we show that *in vivo* treatments modifying apoplastic pH (stem infiltration with a pH buffer) or reducing stem metabolic activity (infiltration with sodium vanadate and sodium cyanide; plant exposure to carbon monoxide) can reduce sugar accumulation, thus disrupting or delaying the recovery process.

- Application of the vanadate treatment ($NaVO_3$, an inhibitor of many ATPases) completely halted recovery from drought-induced embolism for up to 24 h after re-irrigation, while partial recovery was observed *in vivo* in control plants using X-ray microcomputed tomography.

- Our results suggest that stem hydraulic recovery in poplar is a biological, energy-dependent process that coincides with accumulation of sugars in the apoplast during stress. Recovery and damage are spatially coordinated, with embolism formation occurring from the inside out and refilling from the outside in. The outside-in pattern highlights the importance of xylem proximity to the sugars within the phloem to the embolism recovery process.

Introduction

Survival of vascular plants under drought stress is intimately linked to maintaining the functionality of their xylem network. While physical aspects of long-distance water transport in vascular plants and formation/spread of embolism are well understood (Stroock *et al.*, 2014; Jensen *et al.*, 2016), the biology of active recovery from embolism remains hotly debated (Nardini *et al.*, 2011; Brodersen & McElrone, 2013; Knipfer *et al.*, 2016). Groups of researchers think that, in some species, no embolism recovery occurs under natural conditions, (Charrier *et al.*, 2016; Lamarque *et al.*, 2018; Choat *et al.*, 2019), while others assert that recovery is a common process that can take place even under moderate xylem tensions (Salleo *et al.*, 2009; Zwieniecki & Holbrook, 2009; Brodersen *et al.*, 2010; Secchi & Zwieniecki, 2011; Tomasella *et al.*, 2019a). Major controversies originate from the fact that most of the techniques used to study plant hydraulic properties are destructive and have doubted reliability (Cochard

et al., 2013; Hacke *et al.*, 2014). Some techniques may indeed cause artefacts (e.g. increased percentage loss of conductivity (PLC) values) as a result of the excision of xylem under tension, thus potentially allowing for spurious air entry into the conduits even if stems were cut under water (Wheeler *et al.*, 2013; Venturas *et al.*, 2015). Other techniques may cause supersaturation with positive air pressure that might induce embolism and the appearance of its rapid recovery (Wheeler *et al.*, 2013). However, the presence and significance of these artefacts are questioned (Trifilò *et al.*, 2014; Jansen *et al.*, 2015; Scoffoni & Sack, 2015; Ogasa *et al.*, 2016; Nardini *et al.*, 2017; Nolf *et al.*, 2017).

Classical hydraulic techniques for monitoring the presence of xylem embolism are complemented with *in vivo*, nondestructive techniques like magnetic resonance imaging (MRI) (Holbrook *et al.*, 2001; Clearwater & Goldstein, 2005; Wang *et al.*, 2013; Zwieniecki *et al.*, 2013; Fukuda *et al.*, 2015) and X-ray computed microtomography (X-ray micro-CT; Brodersen *et al.*, 2010; McElrone *et al.*, 2013; Choat *et al.*, 2016). These

contemporary techniques make it possible to observe, in real time, the spatial and temporal patterns of embolism occurrence in the hydraulic systems of living plants. While very safe for living cells and capable of fast repetitive imaging, the MRI has relatively low resolution (> 20 μm) and physical limitations on fitting the stem through the core of the magnet. X-ray micro-CT has emerged as the preferred technique for studying xylem embolism formation (Cochard *et al.*, 2015; Savi *et al.*, 2017) and its potential recovery (Brodersen *et al.*, 2010, 2018; Rolland *et al.*, 2015). X-ray micro-CT provides good contrast between air-filled and water-filled conduits, high spatial and temporal resolution (*c.* 1 μm) and high signal-to-noise ratio. A limitation of this technique is that it does not directly measure hydraulic conductivity, but instead allows the number and size of conduits that are air or fluid-filled to be determined, thus limiting ability to calculate the effective conductivity of xylem tissue from the images (Jacobsen & Pratt, 2018; Pratt *et al.*, 2020a,b). A recent study challenged the usefulness of X-ray micro-CT for repeated observations of water content in the same xylem conduits because of the severe damage caused to living cells by consecutive scans (Petruzzellis *et al.*, 2018). Limiting xylem exposure to single scans and reliance on observations of multiple stems might be required to confidently study the hydraulic recovery processes.

Despite these technical difficulties, a growing consensus suggests that, while embolism formation cannot be avoided during severe water stress, recovery might be possible upon relief of stress (lowering tension) and strongly reduced transpiration (Brodersen & McElrone, 2013). To account for this process, several recovery models were proposed (Salleo *et al.*, 2004; Zwieniecki & Holbrook, 2009; Nardini *et al.*, 2011; Brodersen & McElrone, 2013; Secchi & Zwieniecki, 2016; Pagliarani *et al.*, 2019), suggesting that the living parenchyma cells closed to xylem are directly involved in supplying the water, energy and osmotica needed to repair embolized vessels. During drought, soluble sugar content (mostly sucrose) is proposed to increase in living parenchyma cells as a result of elevated starch degradation rates and the necessity of lowering cell osmotic potential in the xylem (Salleo *et al.*, 2009; Secchi & Zwieniecki, 2011, 2016). Increased sugar concentrations in living parenchyma cells trigger sucrose efflux to the apoplast via sucrose transporters. Local concentrations of sugar might be supplemented by sugars supplied from the phloem, decreasing reliance on locally stored starch (Nardini *et al.*, 2011). Sugars and ions accumulated in the apoplast can generate up to *c.* 0.2 MPa osmotic pressure in nonfunctional vessels (Secchi & Zwieniecki, 2012), thus building up an osmotic gradient that allows for cell-by-cell refilling against low tension (Zwieniecki & Holbrook, 2009). *In vivo* observations from both MRI and X-ray micro-CT studies confirm that water may return to empty vessels if a significant reduction in stress occurs (Holbrook *et al.*, 2001; Scheenen *et al.*, 2007; Zwieniecki *et al.*, 2013; Brodersen *et al.*, 2018), and that water droplets preferentially form and grow on the vessel walls that are in contact with living parenchyma cells (Brodersen *et al.*, 2010).

The efflux of sugars is induced by low apoplastic pH conditions that promote the activity of acidic invertases. In a low pH environment, acidic invertases splice sucrose to glucose and fructose, thus reducing the concentration of extracellular sucrose and generating a sucrose gradient between living parenchyma cells and the apoplast, promoting further sucrose efflux from parenchyma. Simultaneously, acidic invertase activity results in the accumulation of monosaccharides in xylem sap, doubling the osmotic potential contributed by sucrose. Active pH adjustment has been confirmed in poplar, where, as predicted by theoretical models, drought induces a pH decrease in the apoplast, causing sugar accumulation in the xylem (Secchi & Zwieniecki, 2016). These stress-related physiological activities are closely coupled to upregulation of genes involved in starch digestion, maltose and sucrose transport and encoding of acidic invertases (Pagliarani *et al.*, 2019). All these observed physiological and transcriptional events are consistent with the priming of xylem for the recovery process. Still required to settle the embolism debate are *in vivo* observations of xylem embolism and recovery, paired with experimental perturbation of xylem chemistry.

Although successful hydraulic recovery necessitates the activity of living parenchyma cells near the xylem, the direct involvement of those cells in this process has not yet been demonstrated. To verify the living parenchyma cell contribution, we perturbed stem biological activity while concurrently visualizing the hydraulic recovery process. We hypothesized that, if sap acidification represents a symptom/signal of severe water stress and if pH-driven sugar accumulation primes stems for embolism recovery when stress is relieved, then inhibition of the biological activity of parenchyma cells during stress will limit, or entirely halt, the hydraulic recovery process. To test this hypothesis, we used X-ray micro-CT observations of poplar stems under stress and post-rehydration in combination with treatments inhibiting the metabolic activity of the living parenchyma cells. Our findings reveal that: poplar trees can reduce embolism extent following water stress relief; embolism formation and disappearance are spatially coordinated, with embolisms accumulating from the inside out, and recovery occurring from the outside in; and experimental reduction of the metabolic activity of dehydrated plants significantly impedes the removal of drought-induced embolisms.

Material and Methods

Plant material and growth conditions

Four month-old hybrid poplars (*Populus tremula* × *Populus alba* clone 717-1B4) were initially grown in a glasshouse at the University of Turin under partially controlled climatic conditions. The glasshouse air temperature and relative humidity averaged *c.* 22°C and 55%, respectively. Maximum photosynthetic photon flux density (PPFD) ranged between 1200 and 1400 μmol photons $m^{-2} s^{-1}$ and 12 h : 12 h, light : dark cycles were followed, using halogen lamps when necessary to supplement light and guarantee a minimum PPFD of 500–600 μmol photons $m^{-2} s^{-1}$. Each plant grew in a 2 l pot filled with a substrate composed of sandy-loam soil, expanded clay, and peat (2 : 1 : 1 by weight). The experiment was conducted on 67 total poplars, c. 50 cm tall with a stem diameter of

3–4 mm. One subgroup of poplars (35 plants) was maintained in the glasshouse at University of Turin; these poplars were used for the chemical manipulations and preliminary analysis of xylem sap. This approach allowed us to determine the timeline of each treatment to optimize time-frame selection for direct X-ray micro-CT observations. A second subset of poplars (32 plants) was moved ahead of the *in vivo* experiment to the glasshouse at the University of Trieste to allow 3 wk of acclimation before the experiments conducted at the Elettra Sincrotrone Trieste facility.

Experimental design

Chemical manipulations (at the University of Turin) In all, 35 plants were used in this study. Five plants were kept as controls (CTR) and watered every day to field capacity. The remaining 30 plants were gradually subjected to water stress (WS) by reducing irrigation until the stem water potential (Ψ_{stem}) was below -1.8 MPa, a value corresponding to at least 50% of PLC (Secchi & Zwieniecki, 2014). Once the target water stress was reached, xylem sap was collected from five plants (stressed, not treated); using a destructive method (Secchi & Zwieniecki, 2012), the other five stressed poplars were rewatered and allowed to recover over the period of 24 h (recovered, not treated, infiltrated with distilled water). After 1 d of stress relief, xylem sap was collected. Before the rewatering phase, the remaining 20 water-stressed poplars were subjected to different chemical manipulations (five plants for each of four treatments) to inhibit the metabolic activity of wood parenchyma cells (Fig. 1a). Four different manipulations were applied:
(**1**) Stem infiltration with distilled water plus sodium orthovanadate ($NaVO_3$; BioLabs, New England, MA, USA), a general inhibitor of many plasma membrane proton pumps, expected to reduce changes in apoplastic pH, decreasing the membrane ATPase transport capacity and thus affecting sucrose transporters. The vanadate solution was used at a concentration of 10 mM.
(**2**) Stem infiltration with distilled water plus sodium cyanide (NaCN; Sigma-Aldrich), to block respiration and, consequently, ATPase activity. The NaCN solution was used at a concentration of 1.0 mM.
(**3**) Stem infiltration with pH 6.5 buffer solution (100 ml of 0.1 M potassium dihydrogen phosphate, 27.8 ml of 0.1 M

sodium hydroxide, 72.2 ml of distilled water), for directly altering apoplastic pH and thus affecting the activity of acidic invertases.
(**4**) Whole-plant exposure to carbon monoxide (CO) gas, for impairing the oxidative respiration and, consequently, ATPase activity.

For stem infiltration, two to three fully expanded leaves, at around one-third tree height, were cut off, leaving the petiole attached to the stem. Then a 2.5-cm-long section of silicon rubber tubing was attached at the remaining petioles and filled with 200 μl of solution (see Fig. 1b). Solutions were allowed to infiltrate the stem via natural stem suction for 2 h. If the absorbed volume exceeded the volume of the solution in the tube, additional liquid was added; on average a total of *c.* 0.75 ml of solution was absorbed into vascular system of each treated plant. Treated plants were allowed 1 d for acclimation, then rewatered and allowed 24 h to recover (recovered, treated) before collecting xylem sap for chemical analyses.

During the carbon monoxide treatment, poplar trees were placed in transparent plastic bags (Fig. 1c). Bags were initially deflated and later filled with CO applied thorough a silicon tube connected to a CO tank until the plastic bag was fully inflated. For the next 3 h, the plants were maintained isolated in the CO-filled bags. After bag removal, treated plants were allowed 1 d of acclimation, then rewatered and allowed 24 h of recovery time (recovered, treated) before xylem sap was extracted.

Plant preparation for X-ray micro-CT observation (at the University of Trieste) The 32 plants used for this part of the study were further divided into two groups: 16 poplars (OV group) to be treated with a sodium orthovanadate solution as described earlier, while the 16 plants belonging to the control group were left untreated. In each group, four plants were kept as unstressed controls and watered every day. The remaining 12 plants were subjected to water stress (< -1.8 MPa). Once plants reached the target water stress, the OV group was treated (as described for Turin experiment). Eight plants from both the control and OV groups were then rewatered. X-ray micro-CT observations were performed on all control, stressed and recovered plants (4 h, until 24 h of recovery time, with only one scan per plant) at Elettra Sincrotrone Trieste, using the SYRMEP beamline (www.elettra.trieste.it; see later for specifics of the X-ray micro-CT observations).
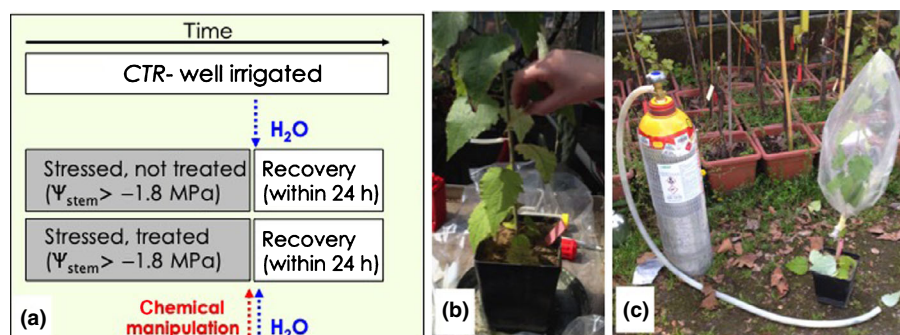

Fig. 1 (a) Schematic representation of experimental setup. (b) Stem infiltration with sodium orthovanadate solution. (c) Plant exposure to carbon monoxide.

Measurements of stem water potential

Stem water potential was measured for each plant on equilibrated nontranspiring (bagged) leaves. Mature leaves were covered with aluminum foil and placed in a humidified plastic bag for at least 30 min before excision. After excision, leaves were allowed to equilibrate for more than 20 min in dark conditions before measuring water potential with a Scholander-type pressure chamber in Turin (Soil Moisture Equipment Corp., Santa Barbara, CA, USA) and with a portable pressure chamber (3005 Plant Water Status Console; Soilmoisture Equipment Corp., Goleta, CA, USA) in Trieste. Stem xylem-pressure changes were monitored for the whole duration of the experiments, from the beginning of the stress treatment until full recovery with varying frequency of days (drying) to hours (recovery).

Sap sampling procedure

Xylem sap from functional vessels was collected from control, stressed, recovered treated and not treated plants according to the method reported in Secchi & Zwieniecki (2012). Sap samples were kept at −20°C until analyses were conducted.

Soluble carbohydrate content and pH measurements

The anthrone-sulfuric acid assay (Leyva et al., 2008) was used to quantify soluble carbohydrate content in xylem sap liquids. The anthrone reagent was prepared immediately before analysis by dissolving 0.1 g of anthrone (0.1%) in 100 ml of concentrated sulfuric acid (98%). Standard solutions were prepared by diluting a Glucose Standard Solution (1.0 mg ml^{-1}; Sigma-Aldrich). We added, 150 μl of anthrone reagent to each well of the microplate containing 50 μl of standard solution, positive control (water), sample solution, and a blank sample. Plates were kept for 10 min at 4°C and then incubated for 20 min at 100°C. After heating, plates were cooled down for 20 min at room temperature before absorbance at 620 nm was read with a microplate reader (Multiscan Thermo Scientific, Waltham, MA USA). Colorimetric response was compared to the glucose standard curve (0, 0.01, 0.03, 0.1 and 0.3 mg l^{-1} glucose) and total carbohydrate content was calculated as mg ml^{-1} glucose.

The pH measurements were taken on sap samples using a micro pH electrode (PerpHect® Ross®; Thermo Fischer Scientific, Waltham, MA, USA).

X-ray Micro-CT observations

Potted poplars were transported to the beamline (see earlier). Before X-ray micro-CT observations, stem water potential was measured on each plant. To reduce sample movement during scan rotation, the whole plant was wrapped in plastic film and secured to a wooden skewer; the pot was then fixed to the beamline sample holder such that stem distance was 10 cm from the detector. The stem was scanned at about 4 cm above the root collar. Two silicon filters (0.5 mm each) were used to obtain an average X-ray source energy of 25 keV, resulting in an entrance dose rate in water of 47 mGy s^{-1}. The X-ray window was 4 mm in height with a horizontal opening up to 120 mm. The exposure time was set at 100 ms, at an angular step of 2° resulting in a 3-min-long scan. During the 360° rotation of the sample, a total of 1600 images were acquired (see Petruzzellis et al., 2018). In total, 32 plants were scanned, and each plant was subjected to only one exposure. After the scan, 14 stems were air-cut a few mm below the scanned section to induce the maximum artificial embolism. Only these samples were then rescanned and analyzed as the others, providing an additional normalization standard for PLC calculations.

In total, 1600 slices per sample with a spatial resolution of 2 μm were reconstructed using the software Syrmep TomoProject (Brun et al., 2015) and one micro-CT slice per sample was analyzed with the ImageJ (1.46r, NIH; https://imagej.nih.gov) software. For each sample, the transverse area of all gas-filled (dark gray) and water-filled (light gray) xylem conduits, the total area of xylem and the distance from embolized vessels to cambium were measured.

The average diameter of each conduit (derived from its area, and assuming a circular shape) was used to calculate the theoretical hydraulic conductivity (K_t) of the xylem, using the Hagen–Poiseuille equation (Tyree & Zimmermann, 2002). The sum of gas-filled (K_{tgas}) and water-filled (K_{twater}) vessel conductivities provided total xylem conductivity (K_{tmax}). The theoretical PLC was then calculated as (K_{tgas}/K_{tmax}) × 100.

Statistical analyses

Significant differences among treatments were tested by one-way ANOVA. The Fisher least significant difference *post hoc* test was used for separating means when ANOVA results were significant ($P < 0.05$). Pairwise differences between treatment means were compared with Student's *t*-test. The Spss statistical software package (v24.0; SPSS Inc., Cary, NC, USA) and Sigmaplot software (Systat Software Inc., San Jose, USA) were used to run the statistical analyses reported earlier and to create figures, respectively.

Results

X-ray micro-CT observations of xylem in intact poplar plants allowed us to distinguish water-filled (functional) from gas-filled (nonfunctional) vessels (Fig. 2a). Almost all vessels in nonstressed plants (stem water potential in the range 0 to −0.5 MPa) were water-filled (Fig. 2b-2). Any higher level of stress (water potential < −0.5 MPa) was associated with an increase in the number of gas-filled conduits (Fig. 2a,b-3). The calculated theoretical conductance of water-filled vessels vs the conductance of all vessels was used to generate a vulnerability curve (PLC vs xylem pressure) and data were fitted to a four-parameter, dose–response logistic curve (Fig. 2a, gray circles and gray lines). While the shape of the obtained curve was similar to typical PLC curves, maximum PLC for severely stressed plants only reached c. 50% (Fig. 2a, red circles), a value lower than that reported previously (Secchi & Zwieniecki, 2014). However, when maximum conductance was determined using only functional vessels (the ones
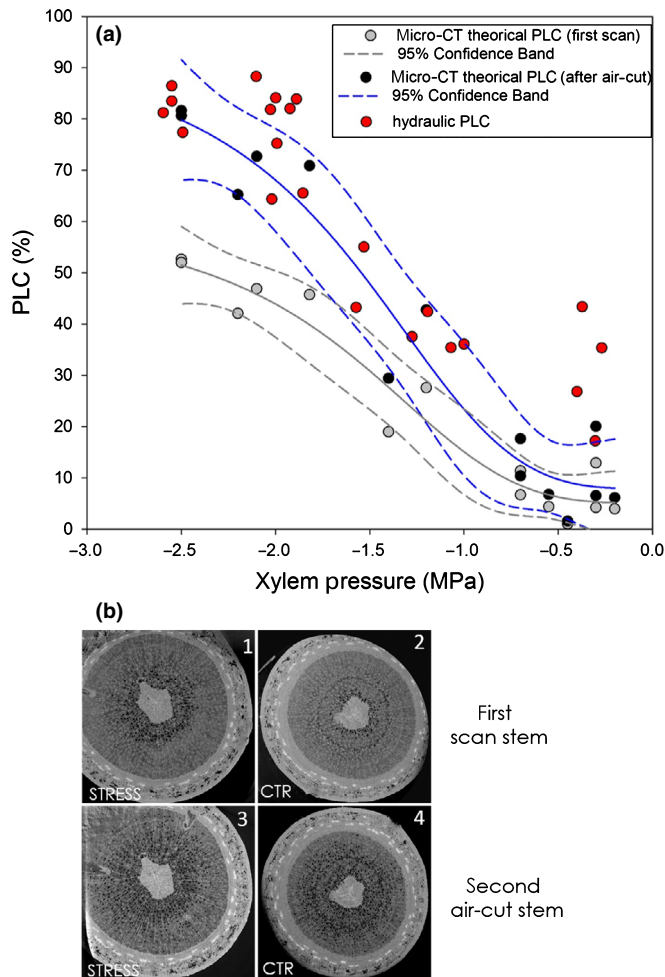
Fig. 2 (a) Vulnerability curves for *Populus tremula* × *Populus alba* plants based on xylem theoretical hydraulic conductivity of plants subjected to one X-ray exposure (gray circles and lines); xylem theoretical hydraulic conductivity normalized with data obtained by stems first air-cut (to induce maximum artificial embolism formation) and then rescanned (black circles and blue lines); hydraulic measurements previously performed on the same poplar clone (red circles; Secchi & Zwieniecki, 2014). Each circle corresponds to a plant. (b) *In vivo* visualization by X-ray microtomography of xylem emboli in stems of *P. tremula* × *P. alba* intact plants. Reconstructed cross-sections showing gas-filled (dark gray) and water-filled (light gray) xylem conduits during well-watered and stress conditions: (1–4) cross-sections of stressed (1) and control (2) stems scanned once; and the same stems (3, 4) exposed to a second exposure after air-cutting. PLC, percentage loss of conductivity.

that embolized after cutting in air), the recalculated PLC matched the previous hydraulic measurements (Secchi & Zwieniecki, 2014), (Fig. 2a, black circles and blue lines). Moreover, the Ψ_{stem} inducing 50% of PLC (P_{50}) was not statistically different between the two vulnerability curves estimates from this study (unadjusted $P_{50} = -1.6$ MPa, gray line; and recalculated PLC $= -1.58$ MPa; Fig. 2a, blue line) and P_{50} (-1.75 MPa; Fig. 2a red circles) reported in the previous study (Secchi & Zwieniecki, 2014).

To facilitate current and future analyses of X-ray micro-CT scans for estimation of embolism extent, we tested the correlation (Supporting Information Fig. S1) between calculated PLC,
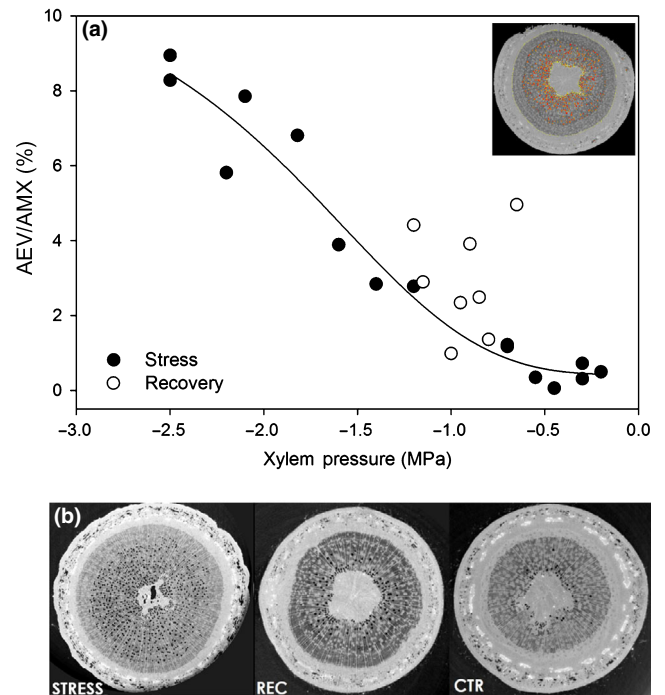

Fig. 3 (a) Percentage of total area of embolized vessels (AEV) over total area of mature xylem (AMX) in response to changes in xylem pressure during drought and recovery treatments. Data were fitted with a four-parameter logistic curve (dose–response curve); each circle corresponds to a plant. (b) *In vivo* visualization by X-ray microtomography (micro-CT) in stems of intact *Populus tremula* × *Populus alba* plants. Reconstructed cross-sections showing embolized (air-filled vessels, dark circles) and functional conduits (water-filled, light gray circles) in stressed, recovered and well-watered plants, respectively.

determined from the diameters of all vessels (see the Material and Methods section), and simple measurements of the total area of embolized vessels (AEV) to total area of mature xylem (AMX; Fig. 3 inset). The correlation was linear with $R^2 = 0.97$ ($N = 14\_P < 0.0001$) allowing for simplified analysis of embolism formation (Fig. S1). Changes in embolism extent using the AEV/AMX ratio ranged from c. 0 in nonstressed plants to $7.72 \pm 1.35\%$ in stressed poplars, with a Ψ_{stem} of -2.32 ± 0.21 MPa, and an EC_{50} of c. -1.92 MPa (we used EC_{50} to describe a 50% change over the range of observed values, not a true change in conductivity) when fitted with a four-parameter logistic curve (Fig. 3a, black circles). The extent of embolism in plants that underwent water stress treatments to levels < -2.0 MPa, and were subsequently rewatered and allowed to recover for several hours (Ψ_{stem} -0.93 ± 0.18 MPa), was $2.92 \pm 0.14\%$, significantly lower than the extent determined for stressed plants that did not recover ($P < 0.0001$; Fig. 3a). This reduction in the AEV/AMX ratio suggests that plants recovering from water stress have fewer embolized xylem conduits than they had before rewatering (Fig. 3b). The formation of embolisms and their disappearance followed a specific spatial pattern, with embolism formation beginning near the pith and extending toward the cambium (i.e. inside out). This was confirmed by analysis of the ratio between distance of the closest embolized vessel to cambium (EV-to-C) in each ray parenchyma wedge to
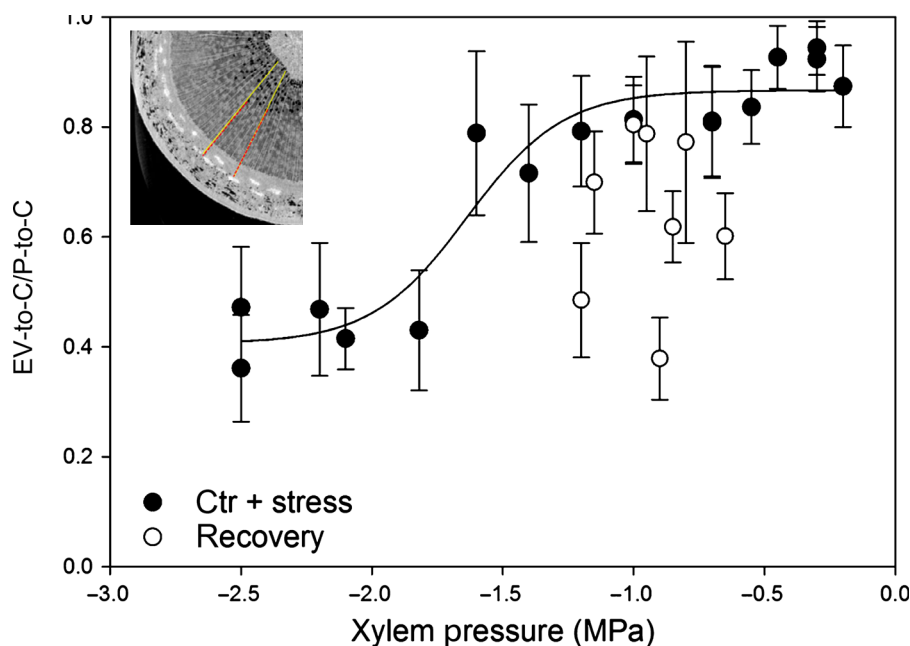
distance between pith and cambium (P-to-C; Fig. 4, black circles). In plants recovering from stress, we observed a significant increase in the average ratio EV-to-C/P-to-C, suggesting that refilling of vessels occurred in the opposite direction to embolization, with regions that embolized last recovering first (outside in; Fig. 4, white circles).

We used three independent approaches to experimentally manipulate the chemistry of xylem sap (pH and content of soluble sugars in sap) during the recovery process. In all cases, and independently of the treatments, plants were capable of recovering water potential to nonstress values within 24 h of rewatering (Fig. 5a). Only $NaVO_3$ and CO treatments were effective in significantly increasing xylem sap pH to c. 6.6, while the control stress was at a pH of c. 5.9 and water infiltration was at a pH of c. 6.2 (one-way ANOVA, $P = 0.001$; Fig. 5b). Treatments with the pH buffer or NaCN did not result in significant changes of xylem sap pH, either as a result of their short-term effects or as a result of the plant's capacity to overcome their presence. High pH values ($NaVO_3$, CO) resulted in low sugar concentrations, while all remaining treatments and stressed plants that had low xylem sap pH had a higher sugar content (Fig. 5c).

We selected the $NaVO_3$ treatment, for its significant impact on pH and the simplicity of its *in vivo* application, to evaluate the impact of metabolic activity on hydraulic recovery, as determined by the presence of embolized vessels. Following the timeline established through our glasshouse experiment, $NaVO_3$ solution was allowed to infiltrate the stems of nonstressed and severely stressed plants (< -2.0 MPa). Subsets of each group were scanned using X-ray micro-CT. The remaining stressed plants were rewatered and allowed adequate time for rehydration (from 4 to 24 h) before scanning. Each plant was scanned only once to avoid X-ray exposure-induced tissue damage. We did not find any significant impact of $NaVO_3$ infiltration on the AEV/AMX

ratio in nonstressed plants, suggesting that treatment with $NaVO_3$ had no effect on xylem native embolism (AEV/AMX ratio ≈ 0.0068; Fig. 6). Similarly, there was no difference in embolism extent between severely stressed nontreated and $NaVO_3$-treated plants (AEV/AMX ratio = respectively 0.072 ± 0.016 and 0.067 ± 0.024; Fig. 6). However, we found a significant effect on AEV/AMX ratio between $NaVO_3$-treated and nontreated plants after several hours of plant rehydration, with treated plants showing a small nonsignificant degree of recovery (change in AEV/AMX from 0.067 ± 0.024 to 0.0534 ± 0.023; Fig. 6), while nontreated plants showed substantial recovery of more than 50% of their conductive capacity (change in AEV/AMX from from 0.072 ± 0.016 to 0.029 ± 0.013; Fig. 6). Finally, there was no difference in recovery of stem water potential (Fig. 6).

Discussion

Combining experimental manipulations of xylem physiochemical status and X-ray micro-CT observations of living plants, we showed that treatments resulting in high apoplastic pH during water stress were detrimental to the accumulation of soluble sugars in xylem, thus significantly reducing the capacity of trees to refill embolized vessels upon recovery from stress without impacting the recovery of stem water potential. Our results verify that recovery of water potential is a nonmetabolic process, and reinforce the idea that embolism refilling — even without water stress — requires biological activity of living parenchyma cells. Direct observations of xylem vessels during recovery from water stress in a high-pH environment support our hypothesis that restoration of xylem transport capacity needs chemical priming. The chemical priming of xylem involves both drop in sap pH and accumulation of sugars in nonfunctional vessels (Secchi & Zwieniecki, 2012).
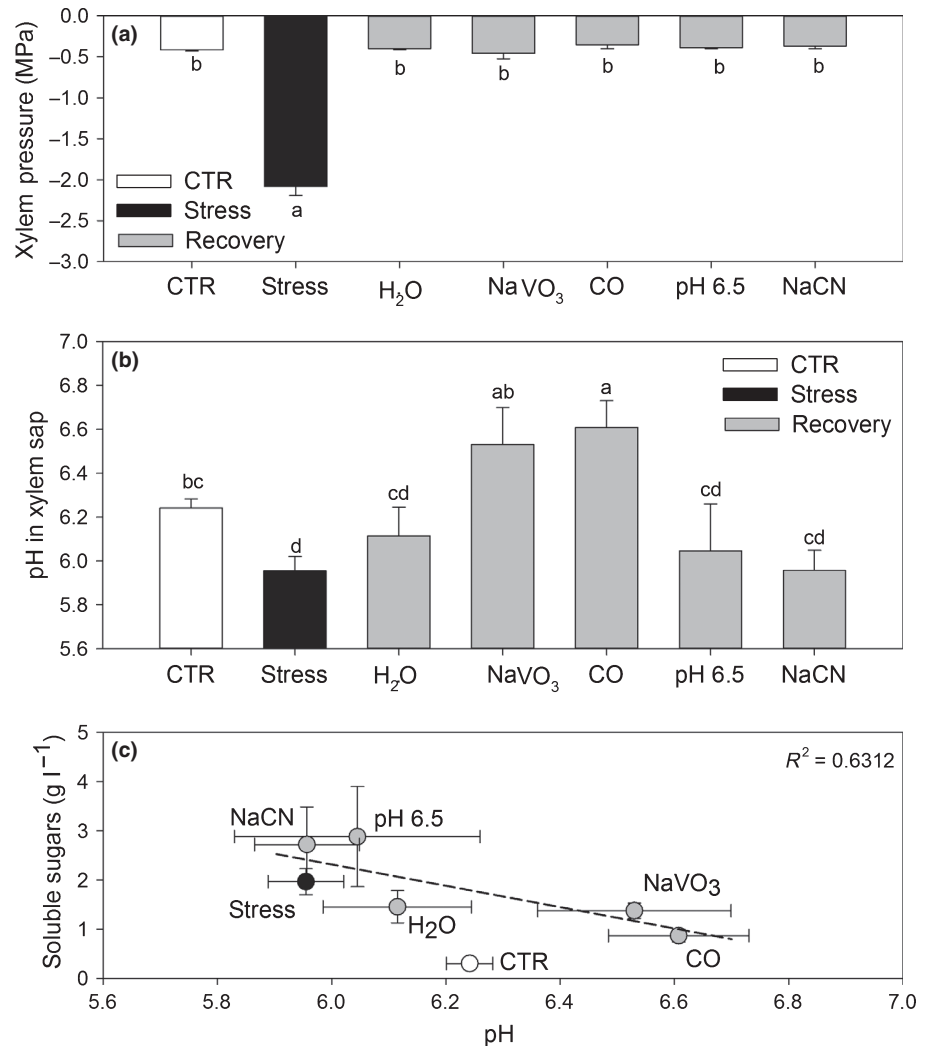
Fig. 5 (a, b) Effect of chemical treatments (sodium orthovanadate, $NaVO_3$; carbon monoxide, CO; pH 6.5 buffer solution; and sodium cyanide, NaCN) on xylem pressure measured on nontranspiring leaves (Ψ_{stem}) (a) and xylem pH (b). (c) Xylem soluble sugar content related to pH values. All plants were water-stressed and then chemically treated, allowing for 1 d acclimation. Poplars were rewatered, and after 24 h of recovery, xylem sap was collected. One-way ANOVA test shows significant differences in xylem pressure ($P < 0.001$), pH values ($P = 0.001$) and sugar content ($P < 0.001$) among different chemical treatments in plants recovering from stress. Letters denote homogeneous groups based on the Fisher least significant difference method; bars are mean values, and error bars represent SE. CTR, control.

In this study, X-ray micro-CT observations were used to determine both the embolism formation during the plant dehydration and the hydraulic recovery following tree rewatering. These *in vivo* analyses confirmed that, when low tension was restored, previously droughted poplar plants recovered from stress by reducing the number of embolized vessels, and potentially reducing PLC. After 4–24 h, poplars repair *c.* 60% of previously embolized conduits. The results of this partial refilling presented here are consistent with xylem hydraulic recovery measured previously on poplars belonging to the same clone, showing that full restoration of stem hydraulic capacity can take several days (Secchi & Zwieniecki, 2014; Pagliarani *et al.*, 2019). Two-dimensional analyses of X-ray micro-CT scans provided detailed information on the propagation of xylem embolisms during dehydration and recovery after irrigation. Initially, embolism occurred in the vessels adjacent to the pith before spreading toward the cambium in correlation with increasing tension. Similar results were reported for *Populus tremula* × *P. alba* (Choat *et al.*, 2016), for *Populus trichocarpa* (Venturas *et al.*, 2019) and for *Vitis vinifera* (Brodersen *et al.*, 2013), where embolisms also form first in the vessels surrounding the pith, and, with the

increase of stress, spread radially towards the cambium within sectors of grouped vessels, via intervessel connections and conductive xylem relays (Brodersen *et al.*, 2013). These previous results show that older vessels are more prone to low-tension embolism formation, potentially suggesting the presence of some degenerative processes that can limit the length of time that vessels function under excessive tension. It could also be possible that older vessels are more susceptible to embolism as a result of cavitation fatigue (Hacke *et al.*, 2001; Stiller & Sperry, 2002), although in our experiment we did not allow plants to be stressed beforehand. Radial embolism propagation, bounded by presence of parenchyma rays, may reflect the occurrence of air seeding from interior vessels toward the outer perimeter, along the path of the greatest vessel-to-vessel contact (Choat *et al.*, 2008).

While the spread of embolism is relatively well documented, much less is known about the spatial dynamics of vessel refilling. We observed recovery of embolized conduits in the opposite direction to their propagation (i.e. outside in, from the cambium towards the pith). Although we did not observe full recovery, the extent of refilling was consistent with expected values given the post-stress stem water potential. In multiple cases, recovery
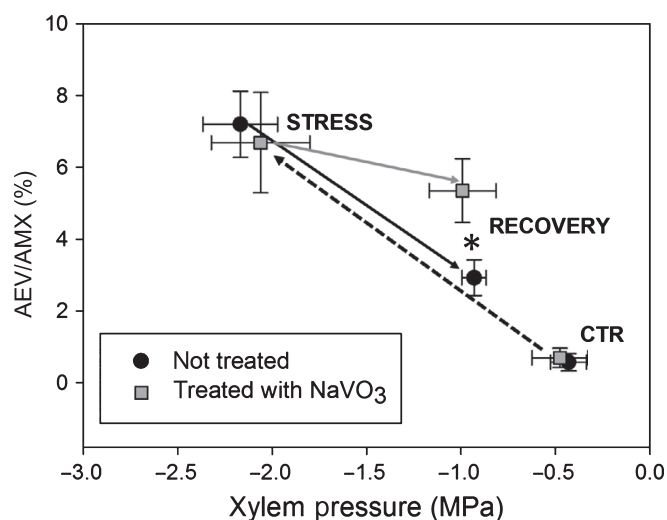
Fig. 6 Percentage of total area of embolized vessels (AEV) over total area of mature xylem (AMX) in response to xylem pressure for non-treated plants (black circles) and for poplars that, before the recovery phase, were chemically treated with a 10 mM sodium orthovanadate solution (light gray squares). Symbols are mean values of multiple embolized vessels belonging to a single plant and error bars represent SD. Asterisk denotes significant differences between treated and nontreated, recovering plants, tested using a t-test ($P < 0.05$).

resulted in a decrease of the average distance between the furthest embolized vessel and the pith, thus suggesting that proximity to cambium is important in providing resources (sugars, ATP and potentially water) for filling embolized vessels. Numerous studies have shown that nonstructural sugars are crucial for maintenance of xylem hydraulic function under water stress (Trifilò et al., 2017; Tomasella et al., 2019b, 2020), and especially for recovery of the hydraulic capacity of the xylem after drought relief (Secchi & Zwieniecki, 2011; Pagliarani et al., 2019; Tomasella et al., 2019a). Theoretical models of embolism removal try to resolve the energy need (Nardini et al., 2011; Secchi & Zwieniecki, 2016; Pagliarani et al., 2019) by proposing that, during water stress, osmotica accumulate in the apoplast in the form of sugars and ions. Direct analysis of xylem sap in embolized vessels indeed supports this view, as both sugars and ions accumulated in nonfunctional vessels can provide an adequate osmotic potential gradient to drain water from parenchyma cells post-recovery (Secchi & Zwieniecki, 2012). Sugars, mostly sucrose derived from starch degradation, are moved from symplast to apoplast through the membrane (passively) or by a proton-coupled sucrose efflux (actively). The accumulation of sugars is controlled by xylem pH, which drops during water stress. A lower pH induces apoplastic sucrose hydrolysis, possibly through acidic invertase activity (Pagliarani et al., 2019), and shifts the sucrose concentration gradient, thereby establishing a further efflux of sucrose to the apoplast. The resulting accumulation of sugar decreases apoplastic water potential, pulling water into the empty vessels upon relief from drought (Salleo et al., 2009; Zwieniecki & Holbrook, 2009, 2012; Secchi & Zwieniecki, 2016). Proton-coupled

sucrose efflux is predicted by models to be responsible for the initial increase in apoplastic sucrose concentration and decrease in pH observed in poplar. The consequent drop in pH triggers an ion efflux from living cells that additionally contributes to apoplastic osmotic concentration (Secchi & Zwieniecki, 2011, 2012). The source of ions might be related to proximity to cambium and phloem, which would be required for recycling of potassium ions to maintain the capacity for this activity (Thompson & Zwieniecki, 2005), further explaining the pattern of refilling from the outside in.

In vitro, it has been shown that in a low-pH environment, sugars continuously accumulate in the xylem apoplast and that this carbohydrate accumulation is significantly reduced in the presence of vanadate, a general proton pump blocker able to inhibit the recycling of protons that are cotransported with sucrose in the apoplast, thus affecting the pH gradient and the sucrose accumulation (Secchi & Zwieniecki, 2011, 2012, 2016). Here, we prove that, when the metabolic activity of stems is decreased, the extent of recovery during rehydration is significantly reduced (Fig. 6). Stem infiltration with vanadate impeded the removal of embolisms formed during drought (only 20% of embolized vessels recovered after stress relief), while a greater extent of embolism removal (c. 60%) was observed in water-treated plants. Similar results were obtained in *Laurus nobilis* L., where stems radially supplied with vanadate did not recover from PLC after 20 min of rehydration to low tension (Salleo et al., 2004). Here we provided a relatively longer water stress relief period (4–24 h) and natural light conditions that encompassed night. @@Despite this prolonged time and a period of no transpiration, embolized vessels remained nonfunctional when metabolic activity had been reduced by vanadate. This lack of recovery is associated with high xylem pH (> 6) and lower soluble sugar content in xylem sap (Fig. 5), suggesting that in the absence of metabolic activity, there was no priming of the stem for recovery, directly linking plant chemistry to visual observations of refilling activity.

The vulnerability curve generated by the X ray micro-CT observations did not closely match the curve based on the classical hydraulic techniques, previously performed on plants belonging to the same poplar clone (Secchi & Zwieniecki, 2014). The *in vivo* observations resulted in underestimation of embolism formation with a maximum of PLC of c. 50%. The underestimate of PLC by micro-CT data was also found in *Populus* in other studies (Jacobsen et al., 2019; Venturas et al., 2019). The discrepancy in PLC values obtained with the two techniques could be attributed to two factors. First, X-ray micro-CT analyses are based on transverse bidimensional reconstructed images of a small scanned segment of stems, and therefore image analysis might miss partially embolized vessels, and thereby overestimate maximum conductance (Loepfe et al., 2007; Pratt & Jacobsen, 2018). These nonfunctional vessels are, however, accounted for in the hydraulic measurements that typically examine much longer stem segments. Second, it is possible that the traditional measurements overestimate the conductive tissue in studied stems, as the outermost layer of xylem may not, as here, show any symptoms of embolisms. The outermost xylem section was also slightly higher in average pixel brightness (i.e. more dense),

suggesting greater hydration of this part of the stem and the possibility that, despite visible vessels, the near-cambial sector may be immature and not yet substantially contribute to axial transport. Underestimation of embolism level, through X-ray micro-CT analysis, was observed previously in *Quercus robur* plants (Choat *et al.*, 2016): the authors suggested the possibility that many of the cells that appeared filled in the images were still living and therefore nonfunctional in transporting water. Pratt & Jacobsen (2018) reported that in grapevine and American chestnut, some vessels commonly observed in the outer growth rings were not contributing to transpiration, and when the samples were dehydrated with air, these vessels showed some deformation, suggesting that they were not yet fully lignified (Pratt & Jacobsen, 2018). In our case, the evidence that vessels located in the outer layer of the xylem were not involved in water transport (or were not experiencing tension) was obtained experimentally by rescanning stem segments that were cut in the air a few mm below the scanned area and allowed to form embolisms as a result of suction in functional vessels. No vessels in the outer layer were ever found to form embolisms. When only mature vessels (the ones that formed embolisms after cutting in the air) were used in the calculation of PLC, the resulting PLC was almost identical to previous data obtained from hydraulic measurements (Fig 2a).

Our results confirm that poplar trees, after rewatering and under low tension, can recover from water stress by reducing the number of embolized vessels in their stems. Further, we provide *in vivo* evidence that refilling is an active, energy-dependent process that relies on metabolically driven xylem sap acidification to accumulate sugars in the apoplast during water stress. By comparing *in vivo* images (without rescanning, which could damage the living parenchyma cells) from two groups of water-stressed plants — with and without experimentally reducing the cellular metabolic activity — we can conclude that refilling is a part of the life of trees (Klein *et al.*, 2018) and that it requires further studies to fully understand how it limits the plant's survival.

Author contributions

FS and MAZ planned and designed the research. FS, CP and MAZ performed the chemical experiments in Turin. FS, CP, SC, FP, TS, G Tromba, AN and MAZ were involved in micro-CT observations. FS, SC, G. Tonel and FP performed the image reconstruction. FS, CP, SC, FP, G Tonel, MMO, CL, AN and MAZ contributed to the analyses and discussion of data. FS, MAZ and AN wrote the manuscript, with contributions and revisions from all other authors.

ORCID

Silvia Cavalletto (iD) https://orcid.org/0000-0001-6478-6936
Claudio Lovisolo (iD) https://orcid.org/0000-0001-8825-2904
Andrea Nardini (iD) https://orcid.org/0000-0002-5208-0087
Maria Margherita Obertino (iD) https://orcid.org/0000-0002-8781-8192
Chiara Pagliarani (iD) https://orcid.org/0000-0003-4656-6192
Francesco Petruzzellis (iD) https://orcid.org/0000-0002-3635-8501
Tadeja Savi (iD) https://orcid.org/0000-0001-7585-763X
Francesca Secchi (iD) https://orcid.org/0000-0002-3161-1643
Giulia Tonel (iD) https://orcid.org/0000-0003-1194-4763
Maciej A. Zwieniecki (iD) https://orcid.org/0000-0002-3774-4455
Giuliana Tromba (iD) https://orcid.org/0000-0002-4576-6099

References

Brodersen CR, Knipfer T, McElrone AJ. 2018. *In vivo* visualization of the final stages of xylem vessel refilling in grapevine (*Vitis vinifera*) stems. *New Phytologist* 217: 117–126.

Brodersen CR, McElrone AJ. 2013. Maintenance of xylem network transport capacity: a review of embolism repair in vascular plants. *Frontiers in Plant Science* 4: 108.

Brodersen CR, McElrone AJ, Choat B, Lee EF, Shackel KA, Matthews MA. 2013. *In vivo* visualizations of drought-induced embolism spread in *Vitis vinifera*. *Plant Physiology* 161: 1820–1829.

Brodersen CR, McElrone AJ, Choat B, Matthews MA, Shackel KA. 2010. The dynamics of embolism repair in xylem. *In vivo* visualizations using high-resolution computed tomography. *Plant Physiology* 154: 1088–1095.

Brun F, Pacile S, Accardo A, Kourousias G, Dreossi D, Mancini L, Tromba G, Pugliese R. 2015. Enhanced and flexible software tools for X-ray computed tomography at the italian synchrotron radiation facility Elettra. *Fundamenta Informaticae* 141: 233–243.

Charrier G, Torres-Ruiz JM, Badel E, Burlett R, Choat B, Cochard H, Delmas CEL, Domec JC, Jansen S, King A *et al.* 2016. Evidence for hydraulic vulnerability segmentation and lack of xylem refilling under tension. *Plant Physiology* 172: 1657–1668.

Choat B, Badel E, Burlett R, Delzon S, Cochard H, Jansen S. 2016. Noninvasive measurement of vulnerability to drought-induced embolism by X-ray microtomography. *Plant Physiology* 170: 273–282.

Choat B, Cobb AR, Jansen S. 2008. Structure and function of bordered pits: new discoveries and impacts on whole-plant hydraulic function. *New Phytologist* 177: 608–625.

Choat B, Nolf M, Lopez R, Peters JMR, Carins-Murphy MR, Creek D, Brodribb TJ. 2019. Non-invasive imaging shows no evidence of embolism repair after drought in tree species of two genera. *Tree Physiology* 39: 113–121.

Clearwater M, Goldstein G. 2005. Embolism repair and long distance transport. In: Holbrook NM, Zwieniecki MA, eds. *Vascular transport in plants*. Oxford, UK: Elsevier, 201–220.

Cochard H, Badel E, Herbette S, Delzon S, Choat B, Jansen S. 2013. Methods for measuring plant vulnerability to cavitation: a critical review. *Journal of Experimental Botany* 64: 4779–4791.

Cochard H, Delzon S, Badel E. 2015. X-ray microtomography (micro-CT): a reference technology for high-resolution quantification of xylem embolism in trees. *Plant, Cell & Environment* 38: 201–206.

Fukuda K, Kawaguchi D, Aihara T, Ogasa MY, Miki NH, Haishi T, Umebayashi T. 2015. Vulnerability to cavitation differs between current-year and older xylem: non-destructive observation with a compact magnetic resonance imaging system of two deciduous diffuse-porous species. *Plant, Cell & Environment* 38: 2508–2518.

Hacke UG, Stiller V, Sperry JS, Pitterman J, McCulloh KA. 2001. Cavitation fatigue. Embolism and refilling cycles can weaken the cavitation resistance of xylem. *Plant Physiology* 125: 779–786.

Hacke UG, Venturas MD, Mackinnon ED, Jacobsen AL, Sperry JS, Pratt RB. 2014. The standard centrifuge method accurately measures vulnerability curves of long-vessel led olive stems. *New Phytologist* 205: 116–127.

Holbrook NM, Ahrens ET, Burns MJ, Zwieniecki MA. 2001. *In vivo* observation of cavitation and embolism repair using magnetic resonance imaging. *Plant Physiology* 126: 27–31.

Jacobsen AL, Pratt RB. 2018. Going with the flow: structural determinants of vascular tissue transport efficiency and safety. *Plant, Cell & Environment* 41: 2715–2717.

Jacobsen AL, Pratt RB, Venturas MD, Hacke UG. 2019. Large volume vessels are vulnerable to water-stress-induced embolism in stems of poplar. *IAWA Journal* 40: 4–22.

Jansen J, Schuldt B, Choat B. 2015. Current controversies and challenges in applying plant hydraulic techniques. *New Phytologist* 205: 961–964.

Jensen KH, Berg-Sorensen K, Bruus H, Holbrook NM, Liesche J, Schulz A, Zwieniecki MA, Bohr T. 2016. Sap flow and sugar transport in plants. *Reviews of Modern Physics* 88: 035007.

Klein T, Zeppel MJB, Anderegg WRL, Bloemen J, De Kauwe MG, Hudson P, Ruehr NK, Powell TL, von Arx G, Nardini A. 2018. Xylem embolism refilling and resilience against drought-induced mortality in woody plants: processes and trade-offs. *Ecological Research* 33: 839–855.

Knipfer T, Cuneo IF, Brodersen CR, McElrone AJ. 2016. *In situ* visualization of the dynamics in xylem embolism formation and removal in the absence of root pressure: a study on excised grapevine stems. *Plant Physiology* 171: 1024–1036.

Lamarque LJ, Corso D, Torres-Ruiz JM, Badel E, Brodribb TJ, Burlett R, Charrier G, Choat B, Cochard H, Gambetta GA *et al.* 2018. An inconvenient truth about xylem resistance to embolism in the model species for refilling *Laurus nobilis* L. *Annals of Forest Science* 75: 88.

Leyva A, Quintana A, Sanchez M, Rodriguez EN, Cremata J, Sanchez JC. 2008. Rapid and sensitive anthrone-sulfuric acid assay in microplate format to quantify carbohydrate in biopharmaceutical products: Method development and validation. *Biologicals* 36: 134–141.

Loepfe L, Martinez-Vilalta J, Pinol J, Mencuccini M. 2007. The relevance of xylem network structure for plant hydraulic efficiency and safety. *Journal of Theoretical Biology* 247: 788–803.

McElrone AJ, Choat B, Parkinson DY, MacDowell AA, Brodersen CR. 2013. Using high resolution computed tomography to visualize the three dimensional structure and function of plant vasculature. *Jove-Journal of Visualized Experiments* 50162.

Nardini A, Lo Gullo MA, Salleo S. 2011. Refilling embolized xylem conduits: Is it a matter of phloem unloading? *Plant Science* 180: 604–611.

Nardini A, Savi T, Losso A, Petit G, Pacile S, Tromba G, Mayr S, Trifilò P, Lo Gullo MA, Salleo S. 2017. X-ray microtomography observations of xylem embolism in stems of *Laurus nobilis* are consistent with hydraulic measurements of percentage loss of conductance. *New Phytologist* 213: 1068–1075.

Nolf M, Lopez R, Peters JMR, Flavel RJ, Koloadin LS, Young IM, Choat B. 2017. Visualization of xylem embolism by X-ray microtomography: a direct test against hydraulic measurements. *New Phytologist* 214: 890–898.

Ogasa MY, Utsumi Y, Miki NH, Yazaki K, Fukuda K. 2016. Cutting stems before relaxing xylem tension induces artefacts in *Vitis coignetiae*, as evidenced by magnetic resonance imaging. *Plant, Cell & Environment* 39: 329–337.

Pagliarani C, Casolo V, Beiragi MA, Cavalletto S, Siciliano I, Schubert A, Gullino ML, Zwieniecki MA, Secchi F. 2019. Priming xylem for stress recovery depends on coordinated activity of sugar metabolic pathways and changes in xylem sap pH. *Plant, Cell & Environment* 42: 1775–1787.

Petruzzellis F, Pagliarani C, Savi T, Losso A, Cavalletto S, Tromba G, Dullin C, Bar A, Ganthaler A, Miotto A *et al.* 2018. The pitfalls of *in vivo* imaging

techniques: evidence for cellular damage caused by synchrotron X-ray computed micro-tomography. *New Phytologist* 220: 104–110.

Pratt RB, Castro V, Fickle JC, Jacobsen AL. 2020a. Embolism resistance of different aged stems of a California oak species (*Quercus douglasii*): optical and microCT methods differ from the benchtop-dehydration standard. *Tree Physiology* 40: 5–18.

Pratt RB, Castro V, Fickle JC, Madsen A, Jacobsen AL. 2020b. Factors controlling drought resistance in grapevine (*Vitis vinifera*, Chardonnay): application of a new microCT method to assess functional embolism resistance. *American Journal of Botany* 107: 618–627.

Pratt RB, Jacobsen AL. 2018. Identifying which conduits are moving water in woody plants: a new HRCT-based method. *Tree Physiology* 38: 1200–1212.

Rolland V, Bergstrom DM, Lenne T, Bryant G, Chen H, Wolfe J, Holbrook NM, Stanton DE, Ball MC. 2015. Easy come, easy go: capillary forces enable rapid refilling of embolized primary xylem vessels. *Plant Physiology* 168: 1636–1647.

Salleo S, Lo Gullo MA, Trifilò P, Nardini A. 2004. New evidence for a role of vessel-associated cells and phloem in the rapid xylem refilling of cavitated stems of *Laurus nobilis* L. *Plant, Cell & Environment* 27: 1065–1076.

Salleo S, Trifilò P, Esposito S, Nardini A, Lo Gullo MA. 2009. Starch-to-sugar conversion in wood parenchyma of field-growing *Laurus nobilis* plants: a component of the signal pathway for embolism repair? *Functional Plant Biology* 36: 815–825.

Savi T, Miotto A, Petruzzellis F, Losso A, Pacile S, Tromba G, Mayr S, Nardini A. 2017. Drought-induced embolism in stems of sunflower: a comparison of *in vivo* micro-CT observations and destructive hydraulic measurements. *Plant Physiology and Biochemistry* 120: 24–29.

Scheenen TWJ, Vergeldt FJ, Heemskerk AM, Van As H. 2007. Intact plant magnetic resonance imaging to study dynamics in long-distance sap flow and flow-conducting surface area. *Plant Physiology* 144: 1157–1165.

Scoffoni C, Sack L. 2015. Are leaves 'freewheelin'? Testing for a Wheeler-type effect in leaf xylem hydraulic decline. *Plant, Cell & Environment* 38: 534–543.

Secchi F, Zwieniecki MA. 2011. Sensing embolism in xylem vessels: the role of sucrose as a trigger for refilling. *Plant, Cell & Environment* 34: 514–524.

Secchi F, Zwieniecki MA. 2012. Analysis of xylem sap from functional (nonembolized) and nonfunctional (embolized) vessels of *populus nigra*: chemistry of refilling. *Plant Physiology* 160: 955–964.

Secchi F, Zwieniecki MA. 2014. Down-regulation of plasma intrinsic Protein1 aquaporin in poplar trees is detrimental to recovery from embolism. *Plant Physiology* 164: 1789–1799.

Secchi F, Zwieniecki MA. 2016. Accumulation of sugars in the xylem apoplast observed under water stress conditions is controlled by xylem pH. *Plant, Cell & Environment* 39: 2350–2360.

Stiller V, Sperry JS. 2002. Cavitation fatigue and its reversal in sunflower (*Helianthus annuus* L.). *Journal of Experimental Botany* 53: 1155–1161.

Stroock AD, Pagay VV, Zwieniecki MA, Holbrook NM. 2014. The physicochemical hydrodynamics of vascular plants. *Annual Review of Fluid Mechanics* 46: 615–642.

Thompson M, Zwieniecki MA. 2005. The role of potassium in long distance transport in plants. In: Holbrook NM, Zwieniecki MA, eds. *Vascular transport in plants*. Oxford, UK: Elsevier, 221–240.

Tomasella M, Casolo V, Aichner N, Petruzzellis F, Savi T, Trifilò P, Nardini A. 2019a. Non-structural carbohydrate and hydraulic dynamics during drought and recovery in *Fraxinus ornus* and *Ostrya carpinifolia* saplings. *Plant Physiology and Biochemistry* 145: 1–9.

Tomasella M, Nardini A, Hesse BD, Machlet A, Matyssek R, Haberle KH. 2019b. Close to the edge: effects of repeated severe drought on stem hydraulics and non-structural carbohydrates in European beech saplings. *Tree Physiology* 39: 717–728.

Tomasella M, Petrussa E, Petruzzellis F, Nardini A, Casolo V. 2020. The possible role of non-structural carbohydrates in the regulation of tree hydraulics. *International Journal of Molecular Sciences* 21: 144.

Trifilò P, Casolo V, Raimondo F, Petrussa E, Boscutti F, Lo Gullo MA, Nardini A. 2017. Effects of prolonged drought on stem non-structural carbohydrates content and post-drought hydraulic recovery in *Laurus nobilis* L.: the possible link between carbon starvation and hydraulic failure. *Plant Physiology and Biochemistry* 120: 232–241.

Trifilò P, Raimondo F, Lo Gullo MA, Barbera PM, Salleo S, Nardini A. 2014.
Relax and refill: xylem rehydration prior to hydraulic measurements favours
embolism repair in stems and generates artificially low PLC values. *Plant, Cell
& Environment* **37**: 2491–2499.

Tyree MT, Zimmermann MH. 2002. *Xylem structure and the ascent of sap, 2nd
edn.* Berlin, Germany: Springer-Verlag.

Venturas MD, Mackinnon ED, Jacobsen AL, Pratt RB. 2015. Excising stem
samples underwater at native tension does not induce xylem cavitation. *Plant,
Cell & Environment* **38**: 1060–1068.

Venturas MD, Pratt RB, Jacobsen AL, Castro V, Fickle JC, Hacke UG. 2019.
Direct comparison of four methods to construct xylem vulnerability curves:
Differences among techniques are linked to vessel network characteristics.
Plant, Cell & Environment **42**: 2422–2436.

Wang MT, Tyree MT, Wasylishen RE. 2013. Magnetic resonance imaging of
water ascent in embolized xylem vessels of grapevine stem segments. *Canadian
Journal of Plant Science* **93**: 879–893.

Wheeler JK, Huggett BA, Tofte AN, Rockwell FE, Holbrook NM. 2013.
Cutting xylem under tension or supersaturated with gas can generate PLC and
the appearance of rapid recovery from embolism. *Plant, Cell & Environment*
36: 1938–1949.

Zwieniecki MA, Holbrook NM. 2009. Confronting Maxwell's demon:
biophysics of xylem embolism repair. *Trends in Plant Science* **14**: 530–534.

Zwieniecki MA, Melcher PJ, Ahrens ET. 2013. Analysis of spatial and temporal
dynamics of xylem refilling in *Acer rubrum* L. using magnetic resonance
imaging. *Frontiers in Plant Science* **4**: 265.

Supporting Information

Additional Supporting Information may be found online in the
Supporting Information section at the end of the article.

Fig. S1 Correlation between imaging-calculated PLC and
embolized vessel surface area weighted by total xylem area.

Please note: Wiley Blackwell are not responsible for the content
or functionality of any Supporting Information supplied by the
authors. Any queries (other than missing material) should be
directed to the *New Phytologist* Central Office.

About *New Phytologist*

- *New Phytologist* is an electronic (online-only) journal owned by the New Phytologist Trust, a **not-for-profit organization** dedicated
  to the promotion of plant science, facilitating projects from symposia to free access for our Tansley reviews and Tansley insights.

- Regular papers, Letters, Research reviews, Rapid reports and both Modelling/Theory and Methods papers are encouraged.
  We are committed to rapid processing, from online submission through to publication 'as ready' via *Early View* – our average time
  to decision is <26 days. There are **no page or colour charges** and a PDF version will be provided for each article.

- The journal is available online at Wiley Online Library. Visit **www.newphytologist.com** to search the articles and register for table
  of contents email alerts.

- If you have any questions, do get in touch with Central Office (np-centraloffice@lancaster.ac.uk) or, if it is more convenient,
  our USA Office (np-usaoffice@lancaster.ac.uk)

- For submission instructions, subscription and all the latest information visit **www.newphytologist.com**