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

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(Article begins on next page)

1 **X-ray micro CT analyses of embolism formation and impact of cellular activity on xylem**  
2 **recovery from stress in poplar trees**

3

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23

24

25 **Summary**

26 In drought stressed plants a coordinated cascade of chemical and transcriptional adjustments  
27 occurs concurrently to embolism formation. While these processes do not affect embolism  
28 formation during stress, they may prime stems for recovery during rehydration by modifying  
29 apoplast pH and increasing sugar concentration in the xylem sap.

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

34 Application of the vanadate treatment ( $\text{NaVO}_3$ , an inhibitor of many ATP-ases) completely  
35 halted recovery from drought-induced embolism for up to 24 hours after re-irrigation, while  
36 partial recovery was observed *in vivo* in control plants using X-ray micro-CT.

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

42

43 Key words: apoplastic pH, embolism, *Populus*, recovery, sugars, X-ray micro-computed  
44 tomography (micro-CT), vanadate, xylem

45

## 46 **Introduction**

47 Survival of vascular plants under drought is intimately linked to maintaining the  
48 functionality of their xylem network. While physical aspects of long-distance water transport in  
49 vascular plants and formation/spread of embolism are well understood (Stroock *et al.*, 2014;  
50 Jensen *et al.*, 2016), the biology of active recovery from embolism remains hotly debated  
51 (Nardini *et al.*, 2011; Brodersen & McElrone, 2013; Knipfer *et al.*, 2016). Groups of researchers  
52 think that, in some species, no embolism recovery occurs under natural conditions (Charrier *et al.*,  
53 2016; Lamarque *et al.*, 2018; Choat *et al.*, 2019) while others assert that recovery is a  
54 common process that can take place even under moderate xylem tensions (Salleo *et al.*, 2009;  
55 Zwieniecki & Holbrook, 2009; Brodersen *et al.*, 2010; Secchi & Zwieniecki, 2011; Tomasella *et al.*,  
56 2019a). Major controversies originate from the fact that the most of the techniques used to  
57 study plant hydraulic properties are destructive, and with doubted reliability (Cochard *et al.*,  
58 2013). Some techniques could indeed cause artefacts (e.g. increased percent loss of conductivity  
59 (PLC) values) due to the excision of xylem under tension, thus potentially allowing for spurious  
60 air entry into the conduits even if stems were cut under water (Wheeler *et al.*, 2013). Other  
61 techniques can cause supersaturation with positive air pressure that could induce embolism and  
62 the appearance of its rapid recovery. However, the presence and significance of these artefacts  
63 are questioned (Trifilo *et al.*, 2014; Fukuda *et al.*, 2015; Scoffoni & Sack, 2015; Ogasa *et al.*,  
64 2016; Nardini *et al.*, 2017; Nolf *et al.*, 2017).

65 Classical hydraulic techniques for monitoring the presence of xylem embolism are  
66 complemented with *in-vivo*, non-destructive techniques like magnetic resonance imaging (MRI)  
67 (Holbrook *et al.*, 2001; Clearwater & Goldstein, 2005; Wang *et al.*, 2013; Zwieniecki *et al.*,  
68 2013) and X-ray computed micro-tomography (X-ray micro-CT; Brodersen *et al.*, 2010;  
69 McElrone *et al.*, 2013; Choat *et al.*, 2016). These contemporary techniques make it possible to  
70 observe, in real time, the spatial and temporal patterns of embolism occurrence in the hydraulic  
71 systems of living plants. The MRI, while very safe for living cells and capable of fast, repetitive  
72 imaging, has relatively low resolution ( $>20\ \mu\text{m}$ ) and physical limitations on fitting the stem  
73 through the core of the magnet. X-ray micro-CT has emerged as the preferred technique for  
74 studying xylem embolism formation (Cochard *et al.*, 2015) and its potential recovery (Brodersen  
75 *et al.*, 2010; Rolland *et al.*, 2015; Brodersen *et al.*, 2018). X-ray micro-CT provides good

76 contrast between air-filled and water-filled conduits, high spatial and temporal resolution (~1  
77  $\mu\text{m}$ ) and high signal-to-noise ratio. However, a recent study challenged the usefulness of X-ray  
78 micro-CT for repeated observations of water content in the same xylem conduits due to the  
79 severe damage caused to living cells by consecutive scans (Petruzzellis *et al.*, 2018). Limiting  
80 xylem exposure to single scans, and reliance on observations of multiple stems, might be  
81 required to confidently study the hydraulic recovery processes.

82 Despite these technical difficulties, a growing consensus suggests, that while embolism  
83 formation cannot be avoided during severe water stress, recovery might be possible upon relief  
84 of stress (lowering tension) and strongly reduced transpiration (Brodersen & McElrone, 2013).  
85 To account for this process, several recovery models were proposed (Salleo *et al.*, 2004;  
86 Zwieniecki & Holbrook, 2009; Nardini *et al.*, 2011; Brodersen & McElrone, 2013; Secchi &  
87 Zwieniecki, 2016; Pagliarani *et al.*, 2019), suggesting that the living parenchyma cells associated  
88 with xylem (vascular associated cells - VACs) are directly involved in supplying the water,  
89 energy and osmotica needed to repair embolized vessels. During drought, soluble sugar content  
90 (mostly sucrose) is proposed to increase in VACs due to elevated starch degradation rates and  
91 the necessity of lowering cell osmotic potential in the xylem (Salleo *et al.*, 2009; Secchi &  
92 Zwieniecki, 2011; Secchi & Zwieniecki, 2016). Increased sugar levels in VACs trigger sucrose  
93 efflux to the apoplast via sucrose transporters. Local levels of sugar might be supplemented by  
94 sugars supplied from the phloem, decreasing reliance on locally stored starch (Nardini *et al.*,  
95 2011). Sugars and ions accumulated in the apoplast can generate up to  $\sim 0.2$  MPa osmotic  
96 pressure in non-functional vessels (Secchi & Zwieniecki, 2012), and thus build-up an osmotic  
97 gradient that allows for cell-by-cell refilling against low tension (Zwieniecki & Holbrook, 2009).  
98 *In vivo* observations from both MRI and X-ray micro-CT studies confirm that water may return  
99 to empty vessels if a significant reduction in stress occurred (Holbrook *et al.*, 2001; Scheenen *et*  
100 *al.*, 2007; Zwieniecki *et al.*, 2013; Brodersen *et al.*, 2018), and that water droplets preferentially  
101 form and grow on the vessel walls that are in contact with VACs (Brodersen *et al.*, 2010).

102 The efflux of sugars is induced by low apoplastic pH conditions that promote the activity  
103 of acidic invertases. In a low pH environment, acidic invertases splice sucrose to glucose and  
104 fructose, thus reducing the concentration of extracellular sucrose and generating a sucrose  
105 gradient between VACs and the apoplast, promoting further sucrose efflux from parenchyma.  
106 Simultaneously, acidic invertase activity results in the accumulation of monosaccharides in

107 xylem sap, doubling the osmotic potential contributed by sucrose. Active pH adjustment has  
108 been confirmed in poplar, where, as predicted by theoretical models, drought induces a pH  
109 decrease in the apoplast, causing sugar accumulation in the xylem (Secchi & Zwieniecki, 2016).  
110 These stress-related physiological activities are closely coupled to upregulation of gene  
111 expressions involved in starch digestion, maltase and sucrose transport and acidic invertases  
112 (Pagliarani *et al.*, 2019). All of these observed physiological and transcriptional events are  
113 consistent with the priming of xylem for the recovery process. Still required to settle the  
114 embolism debate, are *in vivo* observations of xylem embolism and recovery, paired with  
115 experimental perturbation of xylem chemistry.

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

130

## 131 **Material and Methods**

132

### 133 *Plant material and growth conditions*

134 Four month-old hybrid poplars (*Populus tremula* x *Populus alba* clone 717-1B4) were initially  
135 grown in a greenhouse at the University of Turin under partially controlled climatic conditions.  
136 The greenhouse air temperature and relative humidity averaged 22°C and 55% respectively.  
137 Maximum photosynthetic photon flux density (PPFD) ranged between 1200 and 1400  $\mu\text{mol}$

138 photons  $\text{m}^{-2} \text{s}^{-1}$  and 12-h-light/12-h-dark cycles were followed using halogen lamps when  
139 necessary, to supplement light and guarantee a minimum PPFD of 500-600  $\mu\text{mol photons m}^{-2}$   
140  $\text{s}^{-1}$ . Each plant grew in a 2 L pot filled with a substrate composed of sandy-loam soil, expanded  
141 clay, and peat (2:1:1 by weight). The experiment was conducted on 67 total poplars, ~50 cm tall  
142 with a stem diameter of 3 to 4 mm. One sub-group of poplars (35 plants) was maintained in the  
143 greenhouse at University of Turin, these poplars were used for the chemical manipulations and  
144 preliminary analysis of xylem sap. This approach allowed us to determine the timeline of each  
145 treatment to optimize time-frame selection for direct X-ray micro-CT observations. A second  
146 subset of poplars (32 plants) was moved ahead of the *in vivo* experiment to the greenhouse at the  
147 University of Trieste to allow three weeks of acclimation prior to the experiments conducted at  
148 the Elettra Sincrotrone Trieste facility.

149

## 150 *Experimental design*

### 151 (1) *Chemical manipulations (at University of Turin)*

152 35 plants were used in this study. Five plants were kept as controls (*CTR*) and watered every  
153 day to field capacity. The remaining 30 plants were gradually subjected to water stress (*WS*)  
154 by reducing irrigation until the stem water potential ( $\Psi_{\text{stem}}$ ) was below -1.8 MPa, a value  
155 corresponding to at least 50 % of PLC (Secchi & Zwieniecki, 2014). Once the target water-  
156 stress level was reached, xylem sap was collected from five plants (*stressed, not treated*);  
157 using a destructive method (Secchi & Zwieniecki, 2012), the other five stressed poplars were  
158 re-watered and allowed to recover over the period of 24 hours (*recovered, not treated*). After  
159 one day of stress relief, xylem sap was collected. Before the re-watering phase, the  
160 remaining 20 water stressed poplars were subjected to different chemical manipulations (five  
161 plants for each of four treatments) to inhibit the metabolic activity of wood parenchyma cells  
162 (Fig. 1a). Four different manipulations were applied:

163 a) Stem infiltration with distilled water plus sodium orthovanadate ( $\text{NaVO}_3$ , BioLabs, New  
164 England, MA), a general inhibitor of many plasma membrane proton pumps, expected to  
165 reduce changes in apoplastic pH. The vanadate solution was used at a concentration of 10  
166 mM.

167 b) Stem infiltration with distilled water plus sodium cyanide (NaCN, Sigma), to block  
168 respiration and consequently, ATP-ase activity. The NaCN solution was used at a  
169 concentration of 1.0 mM.

170 c) Stem infiltration with pH 6.5 buffer solution (100mL of 0.1 M Potassium dihydrogen  
171 phosphate, 27.8 ml of 0.1M Sodium hydroxide, 72.2 ml of distilled water), for directly  
172 altering apoplastic pH.

173 d) whole plant exposure to carbon monoxide (CO) gas, for impairing the oxidative  
174 respiration and, consequently, ATP-ase activity.

175 For stem infiltration, 2-3 fully expanded leaves, at around 1/3 tree height, were cut-off  
176 leaving petiole attached to the stem. Then a 2.5 cm-long silicon rubber tubing was attached at  
177 the remaining petioles and filled with 200  $\mu$ l of solution (see Fig. **1b**). Solutions were  
178 allowed to infiltrate the stem via natural stem suction for two hours. If the absorbed volume  
179 exceeded the volume of the solution in the tube, additional liquid was added; on average a  
180 total of  $\sim$ 0.75 ml of solution was absorbed into vascular system of each treated plant. Treated  
181 plants were allowed 1-day for acclimation, then re-watered and allowed 24 hours of recovery  
182 time (*recovered, treated*) before xylem sap collected for chemical analyses.

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

## 189 (2) *Plant preparation for X-ray micro-CT observation (at University of Trieste)*

190 The part 32 plants used for this part of the study, were further divided into two groups; 16  
191 poplars (OV group) to be treated with a sodium ortho-vanadate solution as described above,  
192 while the 16 plants belonging to the control group were left untreated. In each group, 4 plants  
193 were kept as unstressed control and watered every day. The remaining 12 plants were  
194 subjected to water stress ( $<$ -1.8 MPa). After plants reach the target water-stress level, the OV  
195 group was treated (as described for Turin experiment). Eight plants from both the control and  
196 OV groups were then re-watered. X-ray micro-CT observations were performed on all



197 control, stressed, and recovered plants (four hours, until 24 hours of recovery time, with only  
198 one scan per plant) at Elettra Sincrotrone Trieste, using the SYRMEP beamline  
199 ([www.elettra.trieste.it](http://www.elettra.trieste.it)), (see below for specifics of X-ray, micro-CT observations)

200

#### 201 *Measurements of stem water potential*

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

211

#### 212 *Sap sampling procedure*

213 Xylem sap from functional vessels was collected from control, stressed, recovered treated and  
214 not treated plants (method in Secchi & Zwieniecki, 2012). Sap samples were kept at -20°C until  
215 analyses were conducted.

216

#### 217 *Soluble carbohydrate content and pH measurements*

218 The anthrone-sulfuric acid assay (Leyva *et al.*, 2008) was used to quantify soluble carbohydrate  
219 content in xylem sap liquids. The anthrone reagent was prepared immediately before analysis by  
220 dissolving 0.1 g of anthrone (0.1%) in 100 mL of concentrated sulfuric acid (98%). Standard  
221 solutions were prepared by diluting a Glucose Standard Solution (1.0 mg/ml; Sigma, Saint Louis,  
222 Missouri, USA). We added, 150 µl of anthrone reagent to each well of the microplate containing  
223 50 µL of standard solutions, positive control (water), sample solutions, and a blank. Plates were  
224 kept for 10 min at 4 °C, then incubated for 20 min at 100 °C. After heating, plates were cooled  
225 down for 20 min at room temperature before absorbance at 620 nm was read with a microplate  
226 reader (Multiscan Thermo Scientific). Colorimetric response was compared to the glucose

227 standard curve (0, 0.01, 0.03, 0.1, and 0.3 mg L<sup>-1</sup> glucose) and total carbohydrate content was  
228 calculated as mg/mL of glucose.

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

231

### 232 *X-ray Micro-CT observations*

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

246 In total, 1600 slices per sample with a spatial resolution of 2 μm were reconstructed using the  
247 software SYRMEP TomoProject (Brun *et al.*, 2015) and one micro-CT slice per sample was  
248 analyzed with the Image J (1.46r, NIH, <https://imagej.nih.gov>) software. For each sample, the  
249 transverse area of all gas-filled (dark grey) and water-filled (light grey) xylem conduits, the total  
250 area of xylem and the distance from embolized vessels to cambium were measured.

251 The average diameter of each conduit (derived from its area, and assuming a circular shape) was  
252 used to calculate the theoretical hydraulic conductivity (K<sub>t</sub>) of the xylem, using the Hagen-  
253 Poiseuille equation (Tyree & Zimmermann, 2002). The sum of gas-filled (K<sub>t<sub>gas</sub></sub>) and water-filled  
254 (K<sub>t<sub>water</sub></sub>) vessel conductivities provided total xylem conductivity (K<sub>t<sub>max</sub></sub>). The theoretical PLC  
255 was then calculated as (K<sub>t<sub>gas</sub></sub>/ K<sub>t<sub>max</sub></sub>) x 100.

256

257 *Statistical analyses*

258 Significant differences among treatments were tested by one-way analysis of variance  
259 (ANOVA). The Fisher LSD post-hoc test was used for separating means when ANOVA results  
260 were significant ( $P < 0.05$ ). Pairwise differences between treatment means were compared with  
261 Student's *t*-test. The SPSS statistical software package (v24.0, SPSS Inc., Cary, NC, USA) and  
262 Sigma Plot software (Systat software Inc., San Jose, USA) were used to run the statistical  
263 analyses reported above and to create figures, respectively.

264

265 **Results**

266 X-ray micro-CT observations of xylem in intact poplar plants allowed us to distinguish water-  
267 filled (functional) and gas-filled (non-functional) vessels (Fig. 2a). Almost all vessels in non-  
268 stressed plants (stem water potential in the range of 0 to -0.5 MPa) were water-filled (Fig. 2b-2).  
269 Any higher level of stress (water potential < -0.5 MPa) was associated with an increase of gas-  
270 filled conduits number (Fig. 2a and 2b-3). The calculated theoretical conductance of water  
271 filled vessels vs. the conductance of all vessels was used to generate a vulnerability curve  
272 (percent loss of conductivity (PLC) versus xylem pressure) and data were fitted to a four-  
273 parameter, dose-response logistic curve (Fig. 2a, grey circles and grey lines). While the shape of  
274 the obtained curve was similar to typical PLC curves, maximum PLC for severely stressed plants  
275 only reached ~50% (Fig. 2a, red circles), a value lower than that reported previously (Secchi &  
276 Zwieniecki, 2014). However, when maximum conductance was determined using only  
277 functional vessels (the ones that embolized after cutting in air), the recalculated PLC matched the  
278 previous hydraulic measurements (Fig. 2a – black circles and blue lines). When subtracting the  
279 baseline PLC value to account for native embolisms, the  $\Psi_{\text{stem}}$  inducing 50% of PLC (P50) was  
280 not statistically different between two estimates from this study (unadjusted EC50: -1.6 MPa,  
281 grey line; and recalculated PLC: -1.58 MPa, Fig. 2a blue line) and P50 (-1.75 MPa; Fig. 2a red  
282 circles) reported in the previous study (Secchi & Zwieniecki, 2014).

283 To facilitate current and future analysis of X-ray micro-CT scans for estimation of  
284 embolism extent, we tested the correlation between calculated PLC, determined from the  
285 diameters of all vessels (see material and methods) with simple measurements of the total area of  
286 embolized vessels (AEV), to total area of mature xylem (AMX; Fig. 3 inset). The correlation was

287 linear with  $R^2=0.97$  ( $N=14$ ,  $p<0.0001$ ) allowing for simplified analysis of embolism formation  
288 (Fig. **1S**). Changes in embolism extent using the AEV/AMX ratio ranged from  $\sim 0$  in non-  
289 stressed plants to  $7.72\% \pm 1.35$  in stressed poplars, with a  $\psi_{\text{stem}}$  of  $-2.32 \pm 0.21$  MPa, and an  
290 EC50 of  $\sim -1.92$  MPa (we used EC50 to describe a 50% change over the range of observed  
291 values, not a true change in conductivity) when fitted with a four-parameter logistic curve (black  
292 circles, Fig. **3a**). Embolism extent in plants that underwent water-stress treatments to levels  
293 below  $-2.0$  MPa, and were subsequently re-watered and allowed to recover for several hours  
294 ( $\psi_{\text{stem}} -0.93 \pm 0.18$  MPa) was  $2.92\% \pm 0.14$ , significantly lower than the extent determined for  
295 stressed plants that did not recover ( $p<0.0001$ ; Fig. **3a**). This reduction in the AEV:AMX ratio  
296 suggests that plants recovering from water stress have fewer embolised xylem conduits than they  
297 did before re-watering. The formation of embolisms and their disappearance followed a specific  
298 spatial pattern, with embolism formation beginning near the pith and extending toward the  
299 cambium (i.e. inside-out). This was confirmed by analysis of the ratio between distance of the  
300 closest embolized vessel to cambium (EV-to-C) in each ray parenchyma wedge to distance  
301 between pith and cambium (P-to-C; Fig. **4**, black circles). In plants recovering from stress, we  
302 observed a significant increase of the average ratio (EV-to-C:P-to-C), suggesting that refilling of  
303 vessels occurred in opposite direction, with regions that embolized last, recovering first (outside-  
304 in; Fig. **4**, white circles).

305 We used three independent approaches to experimentally manipulate the chemistry of  
306 xylem sap (pH and content of soluble sugars in sap) during the recovery process. Our first  
307 approach changed xylem sap pH by infiltrating stems with a pH buffer (pH 6.5), reducing the  
308 activity of acidic invertases. Secondly, we reduced membrane ATP-ase transport capacity by  
309 infiltrating stems with sodium orthovanadate ( $\text{NaVO}_3$ ) solution to disable sucrose transporters.  
310 Our third approach was to reduce respiration by infiltrating stems with sodium cyanide (NaCN)  
311 solution and exposing plants to gaseous carbon monoxide (CO) to reduce the availability of  
312 ATP. As a control we infiltrated stems with DI water. In all cases, and independent of the  
313 treatments, plants were capable of recovering water potential to non-stress levels within 24 hours  
314 of re-watering (Fig. **5a**). Only  $\text{NaVO}_3$  and CO treatments were effective in significantly  
315 increasing xylem sap pH to  $\sim 6.6$ , while the control stress-were at pH  $\sim 5.9$  and water infiltration  
316 at pH  $\sim 6.2$  (ANOVA one-way  $p=0.001$ ; Fig. **5b**). Treatments with a pH buffer or NaCN did not

317 result in significant changes of xylem sap pH, either due to their short-term effects or the plant's  
318 capacity to overcome their presence. High pH values (NaVO<sub>3</sub>, CO) resulted in low sugar  
319 concentrations, while all remaining treatments and stressed plants that had low xylem sap pH had  
320 a higher sugar content (Fig. 5b inset).

321 We selected the NaVO<sub>3</sub> treatment, for its significant impact on pH and the simplicity of  
322 its *in vivo* application, to determine the impact of metabolic activity on hydraulic recovery, as  
323 determined by presence of embolized vessels. Following the timeline established through our  
324 greenhouse experiment, NaVO<sub>3</sub> solution was allowed to infiltrate the stems of non-stressed and  
325 severely-stressed plants (< -2.0 MPa). Subsets of each group were scanned using X-ray micro-  
326 CT. Remaining stressed plants were re-watered and allowed adequate time for rehydration (from  
327 4 to 24 hours) before scanning. Each plant was scanned only once to avoid X-ray exposure  
328 induced tissue damage. We did not find any impact of NaVO<sub>3</sub> infiltration on the AEV/AMX  
329 ratio in non-stressed plants, suggesting that treatment with NaVO<sub>3</sub> had no effect on xylem native  
330 embolism (AEV/AMX ratio = ~0.0068; Fig. 6). Similarly, there was no difference on embolism  
331 extent between severely stressed non-treated, and NaVO<sub>3</sub>-treated plants (AEV/AMX ratio =  
332 respectively  $0.072 \pm 0.016$  and  $0.067 \pm 0.024$ ; Fig. 6). However, we found a significant effect on  
333 AEV/AMX ratio between NaVO<sub>3</sub> treated and non-treated plants after several hours of plant  
334 rehydration, with treated plants showing small non-significant level of recovery (AEV/AMX  
335 ratio change from  $0.067 \pm 0.024$  to  $0.0534 \pm 0.023$ ; Fig. 6), while non-treated plants showed  
336 substantial recovery of more than 50% of their conductive capacity (AEV/AMX ratio change  
337 from  $0.072 \pm 0.016$  to  $0.029 \pm 0.013$ ; Fig. 6), there was no difference in recovery of stem water  
338 potential (Fig. 6).

339

## 340 Discussion

341 Combining experimental manipulations of xylem physiochemical status and X-ray  
342 micro-CT observations of living plants, we show that treatments resulting in high apoplastic pH  
343 during water stress are detrimental to the accumulation of soluble sugars in xylem, significantly  
344 reducing the capacity of trees to refill embolized vessels upon recovery from stress without  
345 impacting the recovery of stem water potential. Our results verify that recovery of water  
346 potential is a non-metabolic process, while reinforcing the idea that embolism refilling – even

347 under without water stress – requires biological activity of VACs. Direct observations of xylem  
348 vessels during recovery from water stress in a high pH environment support our hypothesis that  
349 restoration of xylem transport capacity requires chemical priming. The chemical priming of  
350 xylem involves both drop in sap pH and the accumulation of sugars in non-functional vessels  
351 (Secchi & Zwieniecki, 2012).

352 In this study, X-ray micro-CT observations were used to determine both the embolism  
353 formation during the plant dehydration and the hydraulic recovery following trees re-watering.  
354 These *in vivo* observations confirmed that, when low tension was restored, previously droughted  
355 poplar plants recovered from stress by reducing the number of embolized vessels, and potentially  
356 reducing PLC. After 4 to 24 hours poplars repair ~ 60% of previously embolized conduits. The  
357 results of this partial refilling presented here are consistent with xylem hydraulic recovery  
358 measured previously on poplars belonging to the same clone, showing that full restoration of  
359 stem hydraulic capacity can take several days (Secchi & Zwieniecki, 2014; Pagliarani *et al.*,  
360 2019). Two-dimensional analyses of X-ray micro-CT scans provided detailed information on the  
361 propagation of xylem embolism during dehydration and recovery after irrigation. Initially,  
362 embolism occurred in the primary xylem adjacent to the pith before spreading toward the  
363 cambium in correlation with increasing tension. Similar results were reported for *Populus*  
364 *tremula x alba* clone (Choat *et al.*, 2016) and for *Vitis vinifera* (Brodersen *et al.*, 2013), where  
365 embolisms also form first in the vessels surrounding the pith, and with the increasing stress,  
366 spread radially toward the cambium within sectors of grouped vessels, via inter-vessel  
367 connections and conductive xylem relays (Brodersen *et al.*, 2013). These previous results show  
368 that older vessels are more prone to low-tension embolism formation, potentially suggesting the  
369 presence of some degenerative processes that can limit the length of time that vessels can  
370 function under excessive tension. It could also be possible that older vessels are more susceptible  
371 to embolism due to cavitation fatigue (Hacke *et al.*, 2001; Stiller and Sperry, 2002), although in  
372 our experiment we did not allow plants to get stress prior to experiment. Radial embolism  
373 propagation, bounded by presence of parenchyma rays, may reflect the occurrence of air seeding  
374 from interior vessels toward the outer perimeter, along the path of greatest vessel-to-vessel  
375 contact (Choat *et al.*, 2008).

376 While the spread of embolism is relatively well documented, much less is known about

377 the spatial dynamics of vessel refilling. We observed recovery of embolized conduits in the  
378 opposite direction to their propagation, i.e. outside-in, from the cambium toward the pith.  
379 Although we did not observe full recovery, the extent of refilling was consistent with expected  
380 values given the post-stress stem water potential. In multiple cases, recovery resulted in a  
381 decrease of the average distance between the furthest embolized vessel and the pith, thus  
382 suggesting that proximity to cambium is important in providing resources (sugars, ATP and  
383 potentially water) for filling embolized vessels. Numerous studies have shown that non-structural  
384 sugars are crucial for maintenance of xylem hydraulic function under water stress (Trifilo *et al.*,  
385 2017; Tomasella *et al.*, 2019b; Tomasella *et al.*, 2020), and especially for recovery of the  
386 hydraulic capacity of the xylem after drought relief (Secchi & Zwieniecki, 2011; Pagliarani *et*  
387 *al.*, 2019; Tomasella *et al.*, 2019a). Theoretical models of embolism removal try to resolve the  
388 energy need (Nardini *et al.*, 2011; Secchi & Zwieniecki, 2016; Pagliarani *et al.*, 2019), by  
389 proposing that, during water stress, osmotica accumulate in the apoplast in the form of sugars  
390 and ions. Direct analysis of xylem sap in embolized vessels indeed supports this view, as both  
391 sugars and ions accumulated in non-functional vessels can provide an adequate osmotic potential  
392 gradient to drain water from parenchyma cells post-recovery (Secchi & Zwieniecki, 2012).  
393 Sugars, mostly sucrose derived from starch degradation, are moved from symplast to apoplast  
394 through the membrane (passively) or by a proton-coupled sucrose efflux (actively). The  
395 accumulation of sugars is controlled by xylem pH, which drops during water stress. A lower pH  
396 induces apoplastic sucrose hydrolysis, possibly through acidic invertase activity (Pagliarani *et*  
397 *al.*, 2019), and shifts the sucrose concentration gradient thereby establishing a further efflux of  
398 sucrose to apoplast. The resulting accumulation of sugar decreases apoplastic water potential,  
399 pulling water into the empty vessels upon relief from drought (Salleo *et al.*, 2009; Zwieniecki &  
400 Holbrook, 2009; Secchi & Zwieniecki, 2012; Secchi & Zwieniecki, 2016). Proton-coupled  
401 sucrose efflux is predicted by models to be responsible for the initial increase of apoplastic  
402 sucrose concentration and the decrease in pH, seen in poplar. The consequent drop in pH,  
403 triggers an ion efflux from living cells that additionally contributes to apoplastic osmotic  
404 concentration (Secchi & Zwieniecki, 2011; Secchi & Zwieniecki, 2012). The source of ions  
405 might be related to proximity to cambium and phloem, which would be required for recycling of  
406 potassium ions to maintain the capacity for this activity (Thompson/Holbrook/Zwieniecki),

407 further explaining the pattern of refilling from the outside-in.

408 *In vitro*, it has been shown that in a low-pH environment, sugars continuously  
409 accumulate in the xylem apoplast, and that this carbohydrate accumulation is significantly  
410 reduced in the presence of vanadate, a proton pump blocker (Secchi & Zwieniecki, 2011;  
411 Secchi & Zwieniecki, 2012; Secchi & Zwieniecki, 2016). Here, we prove that, when the  
412 metabolic activity of stems is decreased, the extent of recovery during rehydration is  
413 significantly reduced (Fig. 6). Stem infiltration with vanadate impeded the removal of  
414 embolisms formed during drought (only 20% of embolized vessels recovered after stress relief),  
415 while a greater extent of embolism removal (about 60%) was observed in water-treated plants.  
416 Similar results were obtained in *Laurus nobilis* L., where stems radially supplied with vanadate  
417 did not recover from PLC after 20 min of rehydration to low tension (Salleo *et al.*, 2004). Here  
418 we provided a relatively longer water stress relief period (4 to 24 hours), and natural light  
419 conditions that encompassed night. Despite this prolonged time and a period of no transpiration,  
420 embolized vessels remained non-functional when metabolic activity had been reduced with  
421 vanadate. This lack of recovery is associated with high xylem pH (>6) and lower soluble sugar  
422 content in xylem sap (Fig. 5), suggesting that in the absence of metabolic activity, there was no  
423 priming of the stem for recovery, directly linking plant chemistry to visual observations of  
424 refilling activity.

425 The vulnerability curve generated by the X ray micro-CT observations did not closely  
426 match the curve based on the classical hydraulic techniques, previously performed on plants  
427 belonging to the same poplar clone (Secchi & Zwieniecki, 2014). The *in vivo* observations  
428 resulted in underestimation of embolism formation with a maximum of PLC around 50%. The  
429 discrepancy in PLC values obtained with the two techniques could be attributed to two factors.  
430 First X-ray micro-CT analyses are based on transverse bidimensional reconstructed images of a  
431 small scanned segment of stems, and therefore image analysis may miss partially embolized  
432 vessels, and could thereby overestimate maximum conductance (Loepfe *et al.*, 2007; Pratt &  
433 Jacobsen, 2018). These nonfunctional vessels, are however, accounted for in the hydraulic  
434 measurements that typically examine much longer stem segments. Conversely, it is possible that  
435 these traditional measurements overestimate the conductive tissue in studied stems, as the outer  
436 most layer of xylem may not, as here, show any symptoms of embolisms. The outermost xylem



437 section was also slightly higher in average pixel brightness (i.e., more dense) suggesting greater  
438 hydration of this part of the stem and possibility that, despite visible vessels, the near-cambial  
439 sector may be immature and not yet substantially contribute to axial transport. Underestimation  
440 of embolism level, through X-ray micro-CT analysis, was observed before in *Q. robur* plants  
441 (Choat *et al.*, 2016); the authors suggested the possibility that many of the cells that appeared  
442 filled in the images were still living and therefore non functional in transporting water. Pratt and  
443 Jacobsen (2018) reported that in grapevine and American chestnut, some vessels commonly  
444 observed in the outer growth rings were not contributing to transpiration, and when the samples  
445 were dehydrated with air, these vessels showed some deformation suggesting that they were not  
446 yet fully lignified (Pratt & Jacobsen, 2018). In our case, the evidence that vessels located in the  
447 outer layer of xylem were not involved in water transport (or were not experiencing tension)  
448 were obtained experimentally by rescanning stem segments that were cut in the air few mm  
449 below the scanned area and allowed to form embolism due to suction in functional vessels. No  
450 vessels in the outer layer ever were found to form embolisms. When only mature vessels (the  
451 ones that formed embolisms after cutting in the air) were used in the calculation of PLC, the  
452 resulting PLC was almost identical to previous data obtained from hydraulic measurements (Fig  
453 **2a**).

454 Our results confirm that poplar trees, after re-watering and under low tension, can recover  
455 from water stress by reducing the number of embolized vessels in their stems. Further, we show  
456 that refilling is an active, energy-dependent process that relies on metabolically-driven  
457 acidification to accumulate sugars in the apoplast during water stress. By comparing *in vivo*  
458 images (without rescanning that could damage VACs) from two groups of water-stressed plants  
459 – with and without experimentally reduced metabolic activity – we can conclude that refilling is  
460 a part of the life of trees, and requires further studies to fully understand how it limits stress  
461 survival.

462

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469

#### 470 **Author Contribution**

471 FS and MAZ planned and designed the research. FS, CP and MAZ performed the chemical  
472 experiments in Turin. FS, CP, SC, FP, TS, GT, AN and MAZ were involved in micro CT  
473 observations. FS, SC, GT, FP made the image reconstruction. FS, CP, SC, FP, GT, MMO, CL,  
474 AN and MAZ contributed to the analysis and discussion of data. FS, MAZ and AN wrote the  
475 manuscript, with contribution and revision from all other authors.

476

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625  
626

627 **Figure legends**

628

629 **Fig. 1** (a) Schematic representation of experimental set-up. (b) Stem infiltration with sodium  
630 ortho-vanadate solution. (c) Plant exposure to carbon monoxide.

631

632 **Fig. 2** (a) Vulnerability curves for *Populus alba x tremula* plants based on: xylem theoretical  
633 hydraulic conductivity of plants subjected to one x-ray exposure (grey circles-lines); xylem  
634 theoretical hydraulic conductivity normalized with data obtained by stems first air-cut (to induce  
635 maximum artificial embolism formation) and then re-scanned (black circles and blue lines);  
636 hydraulic measurements previously performed on the same poplar clone (red circles, Secchi and  
637 Zwieniecki 2014). Each circle corresponds to a plant. (b) *In vivo* visualization by X-ray  
638 microtomography of xylem emboli in stems of *Populus tremula x alba* intact plants.  
639 Reconstructed cross sections showing gas-filled (dark grey) and water-filled (light grey) xylem  
640 conduits during well watered and stress conditions. 1-2 cross-sections of stressed and control  
641 stems scanned once and the same stems exposed to a second exposure after air-cutting (3-4).

642

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

650

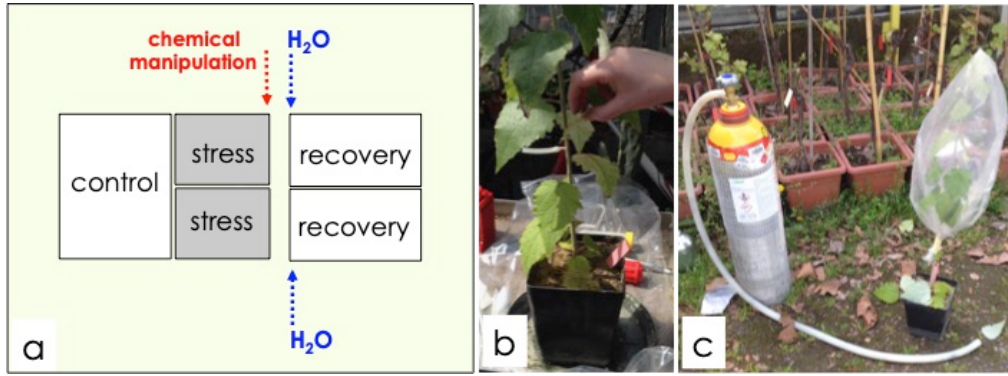
651 **Fig. 4** Ratio between the distance of the closest embolized vessels to cambium (EV-to-C) in each  
652 ray parenchyma wedge to the distance between the pith and cambium (P-to-C) in stressed, well-  
653 watered, and recovered plants. Circles are mean values of multiple embolized vessels belonging  
654 to a single plant and error bars represent SD. Inset: Reconstructed cross section showing distance  
655 from pith to the cambium (yellow lines) and from the closest embolized vessels to cambium (red  
656 dotted lines).



657 **Fig. 5** Effect of chemical treatments (sodium orthovanadate,  $\text{NaVO}_3$ ; carbon monoxide,  $\text{CO}$ ;  
658 pH6.5 buffer solution and sodium cyanide,  $\text{NaCN}$ ) on: (a) Xylem pressure measured on non-  
659 transpiring leaves ( $\Psi_{\text{stem}}$ ). and (b) xylem pH. Inset: average xylem sugar content measured for  
660 each treatment as it relates to average pH values. All plants were water-stressed and then  
661 chemically treated, allowing for 1 day for acclimation. Poplars were re-watered, and after 24  
662 hours of recovery, xylem sap was collected. One-way ANOVA test suggests significant  
663 differences in xylem pressure ( $p < 0.001$ ), pH values ( $p = 0.001$ ) and sugar content ( $p < 0.001$ )  
664 between different chemical treatments in plants recovering from stress. Letters denote  
665 homogeneous groups based on the Fisher LSD method; bars are mean values, and error bars  
666 represent SE.

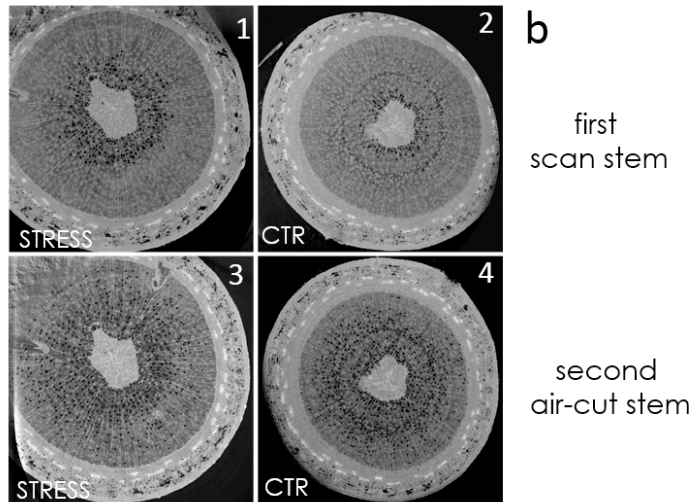
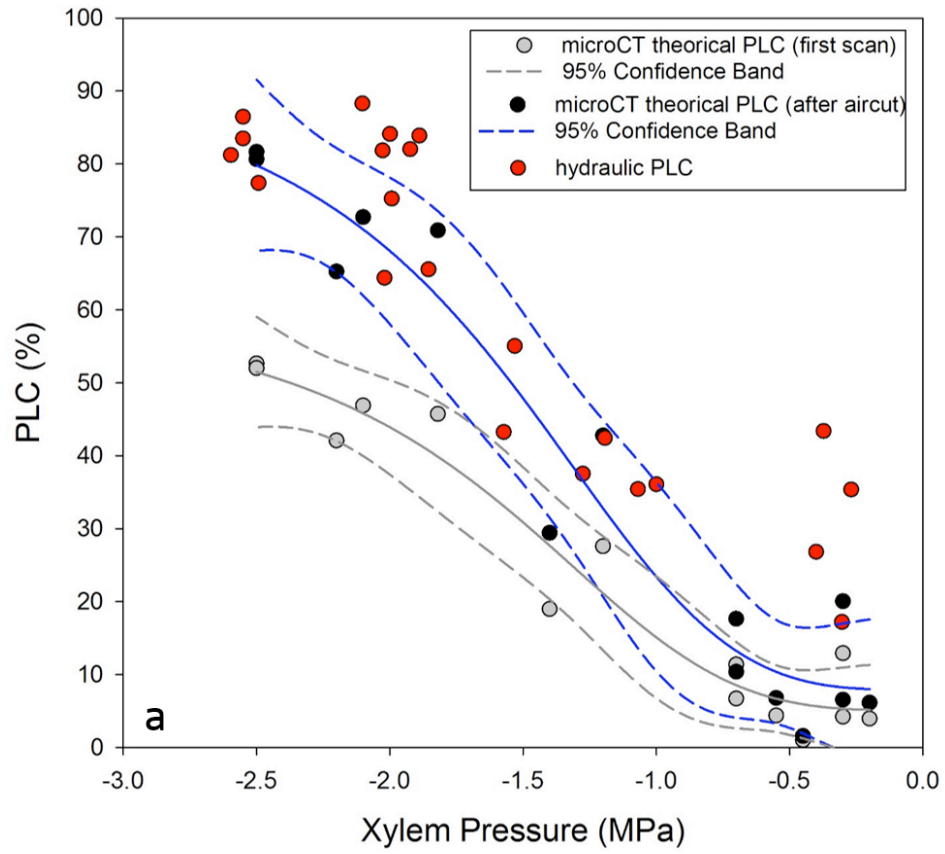
667  
668 **Fig. 6** Percentage of total area of embolized vessels (AEV) on total area of mature xylem (AMX)  
669 in response to xylem pressure for non treated plants (black circles) and for poplar that before the  
670 recovery phase were chemical treated with a solution 10 mM of sodium orthovanadate (light  
671 grey squares). Symbols are mean values of multiple embolized vessels belonging to a single  
672 plant and error bars represent SD. Asterisk denotes significant differences between treated and  
673 non-treated, recovering plants, tested using a t-test ( $p < 0.05$ ).

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676

677 Fig. 1

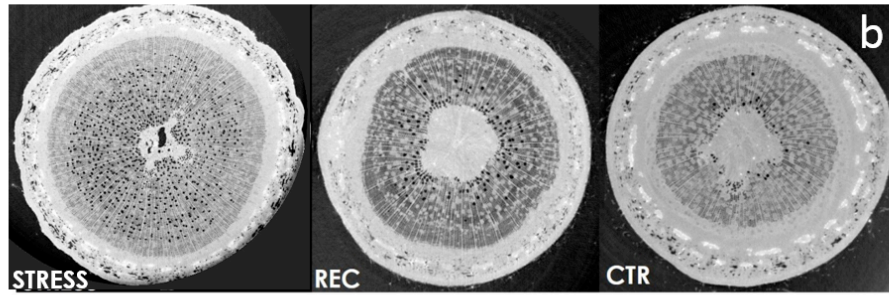
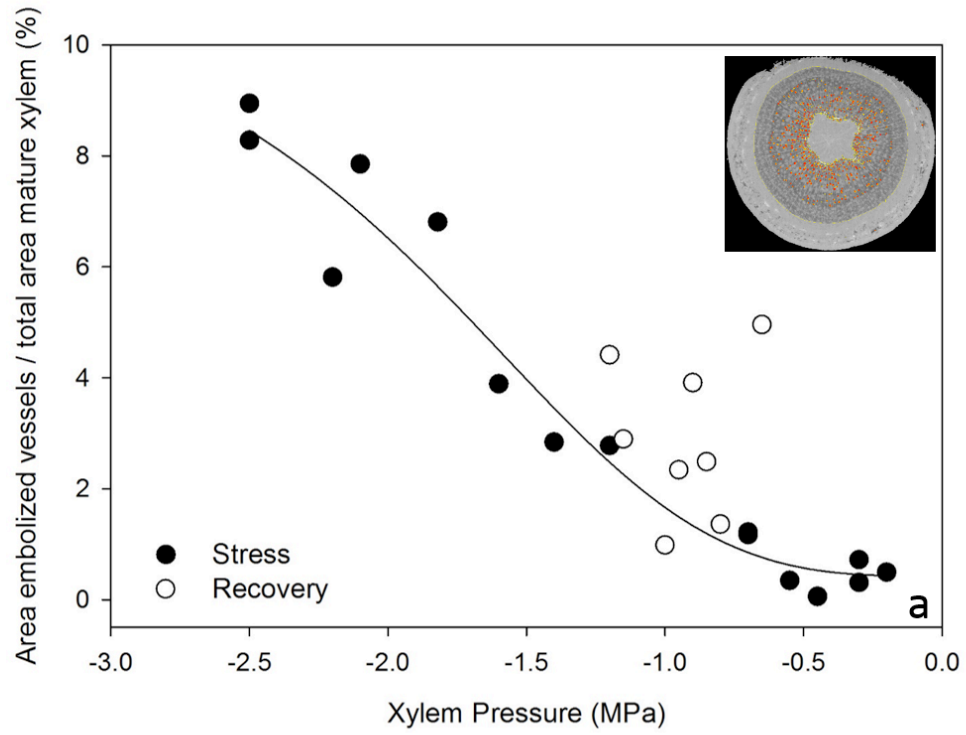


678

679 Fig. 2

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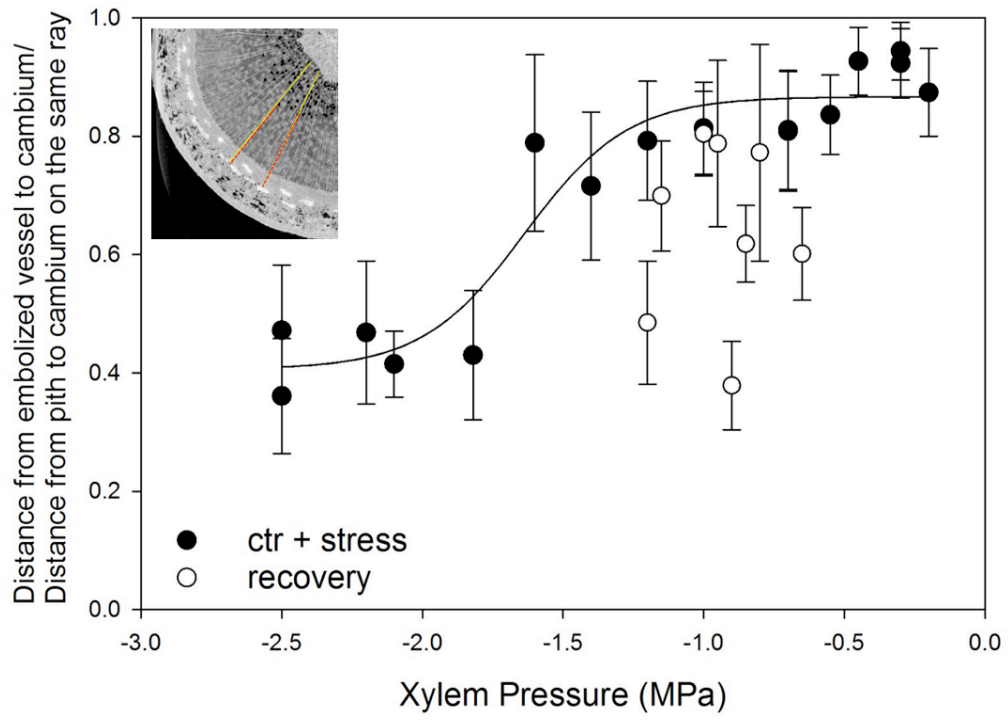


682

683 Fig. 3

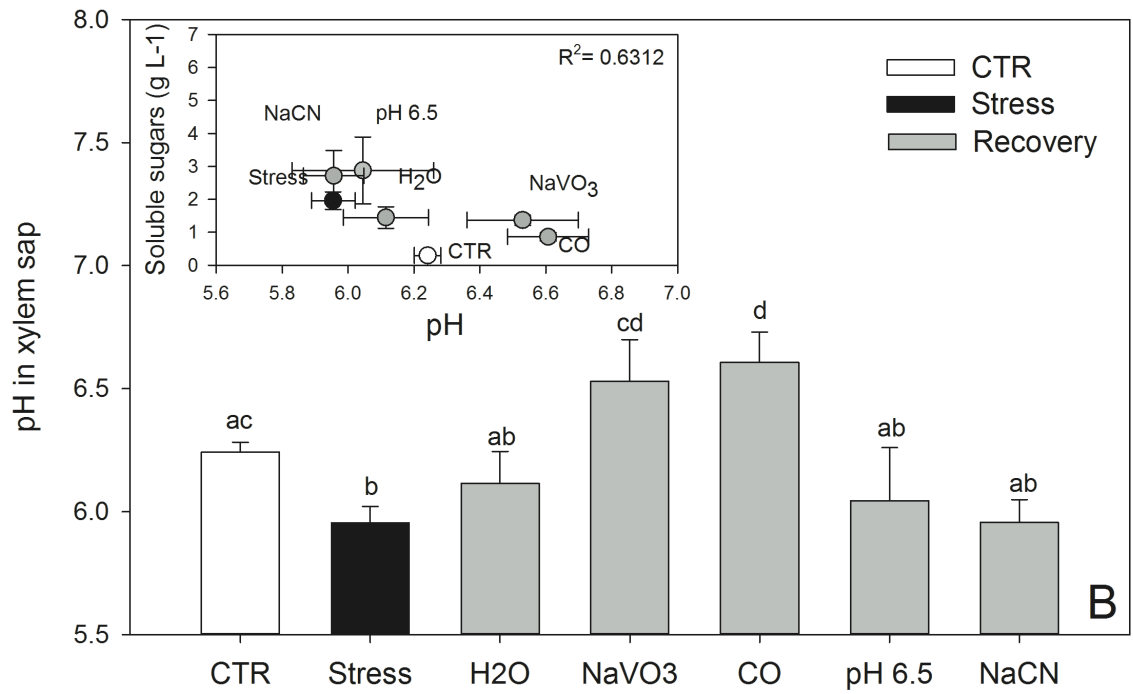
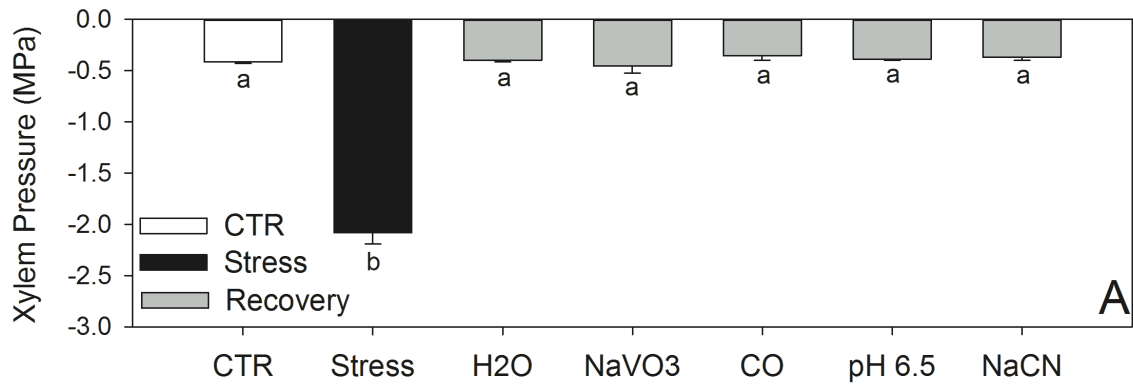
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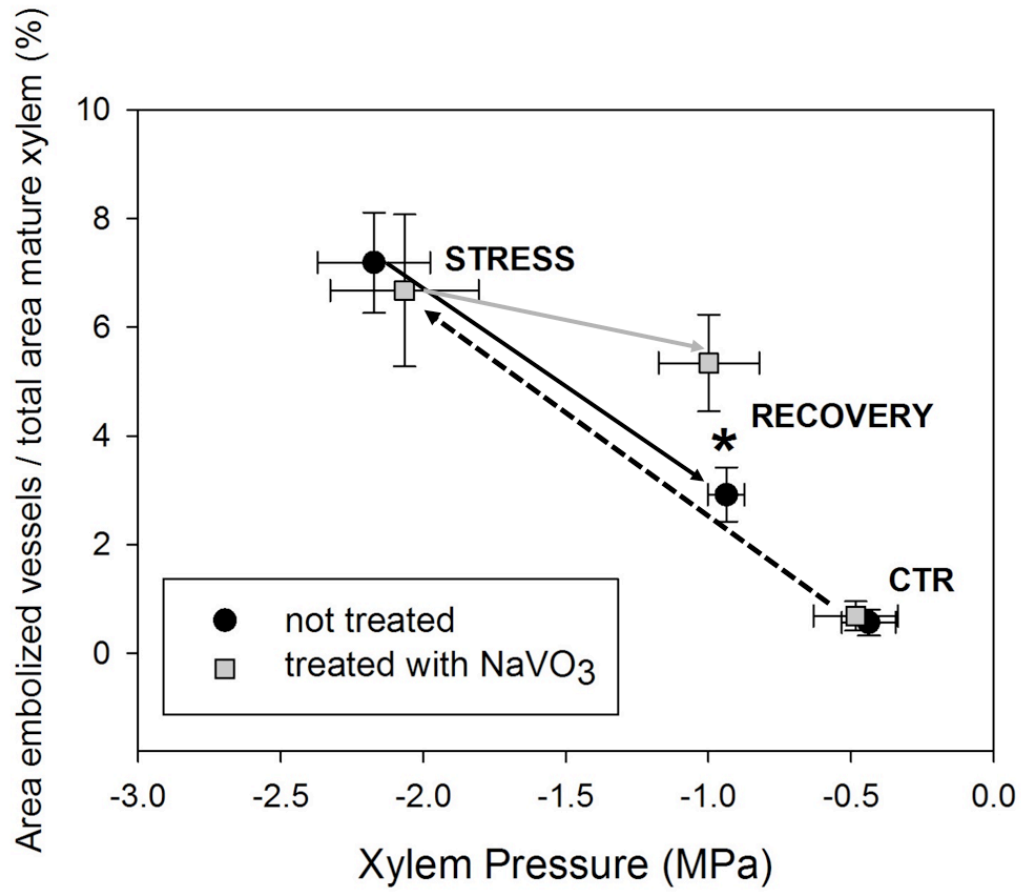
687 Fig. 4



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689 Fig. 5

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691

692 Fig. 6

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