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**Analysis of xylem sap from functional (non-embolized) and non-functional (embolized) vessels of *Populus nigra* - chemistry of refilling**

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1 **Chemistry of xylem embolism refilling.**

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14 **Analysis of xylem sap from functional (non-embolized) and non-functional (embolized)**  
15 **vessels of *Populus nigra* - chemistry of refilling.**

16

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24

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32

33 **Abstract**

34

35         It is assumed that the refilling of drought induced embolism requires the creation of an  
36 osmotic gradient between xylem parenchyma cells and vessel lumens to generate the water  
37 efflux needed to fill the void. To assess the mechanism of embolism repair, it is crucial to  
38 determine if plants can up-regulate the efflux of osmotically active substances into embolized  
39 vessels and identify the major components of the released osmoticum. Here, we introduce a new  
40 approach of sap collection designed to separate water from non-embolized (functional) and  
41 embolized vessels (non-functional). This new approach made possible the chemical analysis of  
42 liquid collected from both types of vessels in plants subjected to different levels of water stress.  
43 The technique also allowed us to determine the water volumes in non-functional vessels as a  
44 function of stress level. Overall, with the increase of water stress in plants, the osmotic potential  
45 of liquid collected from non-functional vessels increased while its volume decreased. Results  
46 revealed the presence of both sugars and ions in non-functional vessels at elevated levels in  
47 comparison to liquid collected from functional vessels, in which only traces of sugars were  
48 found. The increased sugar concentration was accompanied by decreased xylem sap pH. These  
49 results provide new insight into the biology of refilling, underlining the role of sugar and sugar  
50 transporters, and imply that a large degree of hydraulic compartmentalization must exist in the  
51 xylem during the refilling process.

52

## 53 **Introduction**

54

55 Long-distance water transport in vascular plants occurs in conduit network of nonliving  
56 cells connecting roots to leaves (Sperry, 2003). In certain conditions, such as drought and/or high  
57 evaporative demand, the water column within the lumen of xylem vessel or tracheid can be  
58 subjected to tensions that result in cavitation and the subsequent formation of embolism, causing  
59 a decrease in stem hydraulic conductance and a loss of plant productivity (Tyree and Sperry,  
60 1989; Holttta et al., 2009; Zwieniecki and Holbrook, 2009). Plants have evolved several  
61 mechanisms in order to mitigate the loss of the water transport capacity. These include shading  
62 leaves or small branches (shrubs) to lower evaporative demand, generating root pressure (small  
63 herbaceous plants) to refill embolized conduits, or growing new vessels or tracheids to replace  
64 lost capacity (Sperry et al., 1987; Stiller and Sperry, 2002). However, these strategies are limited  
65 in their usefulness, as to be successful they require both relief from water stress/transpiration and  
66 prolonged time. The ability of plants to dynamically refill embolized conduits under adverse  
67 conditions, such as large soil water deficits or high transpiration rates, would allow for greater  
68 flexibility in plants response to water stress and avoid temporal losses to photosynthetic capacity.  
69 How refilling can occur in the presence of large xylem tension has proved to be difficult to  
70 understand (Holbrook and Zwieniecki, 1999; Tyree et al., 1999), and only recently has in vivo  
71 imaging undoubtedly confirmed the ability of plants to refill embolized vessels (Holbrook et al.,  
72 2001; Clearwater and Goldstein, 2005), and that water droplets preferentially are formed on the  
73 vessel walls adjacent to parenchyma cells (Brodersen et al., 2010). However, despite significant  
74 scientific efforts (Salleo et al., 1996; Zwieniecki and Holbrook, 2009; Secchi and Zwieniecki,  
75 2010; Nardini et al., 2011), the mechanism responsible for embolism refilling under negative  
76 pressure is still not well understood.

77 Various studies have proposed and partially confirmed that the refilling process requires a  
78 source of water to fill the empty conduits and a source of energy to overcome existing free-  
79 energy gradients acting against it. Both sources, water and energy, have to be provided by the  
80 adjacent living parenchyma cells, and their role in embolism refilling is confirmed by studies  
81 showing that physical damage to phloem or metabolic inhibition of parenchyma cells in stems  
82 prohibited the recovery process (Salleo et al., 2004; Zwieniecki et al., 2004). If xylem  
83 parenchyma cells supply water for refilling, or at least for part of it, a role for aquaporins in this

84 process can be expected. Studies on walnut (*Juglans regia*) showed that higher expression of two  
85 PIP2 genes (*JrPIP2.1* and *JrPIP2.2*) was observed in vessel-associated parenchyma cells at the  
86 same time that embolism refilling took place (Sakr et al., 2003). Moreover, expression levels of  
87 several PIP1 and PIP2 genes were shown to increase during the refilling process in some other  
88 species, including *P. trichocarpa* and *Vitis vinifera* (Kaldenhoff et al., 2008; Secchi and  
89 Zwieniecki, 2010; Secchi et al., 2011; Perrone et al., 2012). Recently, a detailed full analysis of  
90 the transcriptome in response to the presence of embolism in poplar stems has revealed the  
91 complexity of genetic activity that is associated with the process of refilling. It was shown that  
92 different aquaporin subfamilies were strongly up-regulated during refilling. This up-regulation  
93 may facilitate the release of water volumes to refill the empty vessels during recovery from  
94 embolism (Secchi et al., 2011). While aquaporins allow for water facilitation between  
95 parenchyma cells and xylem they are passive transporters i.e. water flows through them down the  
96 free energy gradient. Thus, to achieve refilling a mechanism for driving water potentials in the  
97 embolized xylem lumens more negative than in the surrounding vascular parenchyma is  
98 required.

99         As the process of embolism refilling under tension is energy demanding, it consequently  
100 requires an adequate supply of carbohydrates to alter the preexisting free energy gradients.  
101 Several studies have demonstrated that the incidence of embolism alters carbohydrate  
102 metabolism and carbon partitioning between starch and soluble sugars in the different tissues, as  
103 well as related enzyme activities and gene expression. Both visualization techniques and  
104 enzymatic analysis of nonstructural carbohydrates showed a decrease in starch content and a  
105 down-expression of the related genes (e.g. amylase) in response to embolism formation.  
106 Furthermore, the drop in starch content was associated with an increase in the level of sucrose in  
107 parenchyma cells (Regier et al., 2009; Salleo et al., 2009; Secchi and Zwieniecki, 2010; Nardini  
108 et al., 2011). These results are strongly supported by a recent transcriptome analysis that found in  
109 response to embolism both down-regulation of genes transcribing for the monosaccharide  
110 metabolic pathways and strong up-regulation of those involved in the disaccharide metabolic  
111 pathways that include starch metabolism (Secchi et al., 2011). However, the role in refilling of  
112 sugars derived from depolymerisation of starch stored in xylem parenchyma is unknown, and  
113 two hypothesis may be proposed: 1) The sugars contribute indirectly to the generation of an ion  
114 efflux into the xylem apoplast via respiration (glucose is converted into pyruvate and the energy



115 released is stored in NADH and ATP that can be used to activate the membrane transporters),  
116 thereby producing the necessary osmotic gradient; or 2) the sugars themselves are transported  
117 out of the parenchyma cells and loaded into cavitated vessels where they directly contribute to  
118 the generation of the osmotic gradients driving water flow from the parenchyma to the embolized  
119 conduits.

120         The transport of sugars between cells and apoplast is mediated by plasma membrane  
121 sugar/proton co-transporters, as energized by membrane H<sup>+</sup>-ATPase. Proton pumps have also  
122 been localized in xylem-associated cells (De Boer and Volkov, 2003) and treatments inducing  
123 their inhibition led to inhibition of xylem refilling (Salleo et al., 2004). Besides being involved in  
124 driving the import/export of sugars between parenchyma and xylem conduits, the proton pumps  
125 are hypothesized to control the apoplastic pH by driving H<sup>+</sup> ions into the sap of well-watered  
126 plants (Sharp and Davies, 2009). Furthermore, the gradient in pH across the cellular membrane  
127 plays an important role in influencing the direction of sugar flow via proton co-transport across  
128 plasma membranes. Alteration in pH is one of the first chemical changes measurable in xylem  
129 sap from plants exposed to drought (Bahrun et al., 2002; Sobeih et al., 2004), and sap  
130 alkalization is often observed in transpiring plants. It is theorized that the increase in sap pH  
131 results in an increase in ABA concentration in transpiring tissues, limiting water loss in drying  
132 soils via stomatal closure. However, increase in xylem sap pH in drought conditions is not a  
133 universal phenomenon. A recent study demonstrates that in woody plants, xylem sap alkalization  
134 is much less common than in the annual species, and of the 22 species studied only 4 showed a  
135 pH increase in sap collected from the transpiration stream (Sharp and Davies, 2009). As sap pH  
136 is an important parameter that can influence sugar transport across cellular membranes,  
137 measuring pH along with sap osmotic properties should yield a better understanding of the  
138 chemistry that drives refilling.

139         One of the big stumbling blocks in gaining insight into the process of refilling is our  
140 inability to characterize the properties of the liquid derived specifically from the vessels that are  
141 being refilled. The volume of this water is very small, and as observed recently randomly  
142 distributed across the stem (Brodersen et al., 2010). Accessing this liquid for analysis presents a  
143 major technical challenge. Here we present a new approach to collecting sap designed to separate  
144 water from *functional* vessels (not embolized) from *non-functional* vessels (embolized that are  
145 being presumably refilled). We then analyze the chemical properties of the xylem sap collected

146 from both populations of vessels, embolized and functional, in plants subjected to different levels  
147 of water stress, with the goal of determining the major components of the osmotic driving force  
148 responsible for embolism refilling.

## 149 150 **Results**

151  
152 *Populus nigra* stems were vulnerable to stress induced embolism. Initial percent loss of  
153 conductivity (PLC) in well watered plants was relatively high, averaging around 50% (Figure 1).  
154 Further increases in PLC were observed with decreasing stem water potential, reaching ~100%  
155 loss below -2.5 MPa. The fitted four parameter logistic curve ('dose response curve') in the  
156 form of  $PLC = min_{PLC} + (max_{PLC} - min_{PLC}) / (1 + (\Psi / EC50)^{slope})$  was constrained with minimum  
157 PLC ( $min_{PLC}$ ) at 49.1% (average of initial PLC values on well watered plants) and maximum  
158 PLC ( $max_{PLC}$ ) at 100%. The resulting response function predicts 50% loss of functional vessels  
159 ( $EC50$ ) at -1.22 MPa (SD=0.1068, t=11.42 and p<0.0001) with relatively slow phase of increase  
160 in PLC i.e.  $slope = -2.27 [MPa^{-1}]$  (SD=0.53, t=4.22 and p=0.0003). The fit was statistically  
161 significant ( $R^2 = 0.74$  and p<0.0001; Figure 1). Relief from water stress resulted in significant  
162 increase in stem water potential over two hours to pre-stress levels, but recovery of PLC was  
163 highly variable with moderately stressed plants ( $-2.0 < \Psi < 1.2$  MPa) showing a significant drop  
164 in PLC over a period of 2.5 hours from average of 75% to 54% (t-value=4.52, df=12, p<0.001).  
165 PLC in recovered plants was not significantly different from that observed in never stressed  
166 plants respectively 54% and 51% (t-value=0.67, df=14, p=0.51). Severely stressed plants ( $\Psi < -$   
167 2.0 MPa) also showed significant recovery in PLC from 97% to 82% (t-value=10.8, df=5,  
168 p<0.001) but these plants did not recover to pre-stress values.

169 The volume of water in non-functional vessels was negatively correlated with stem water  
170 potential. Non-functional volume was in the range of 5 to 20% of total vessel volume in well  
171 watered and low stressed plants, and dropped dramatically to under 10 % in moderately stressed  
172 plants. Only very small volumes of water were collected from non-functional conduits of plants  
173 stressed to below -2.0 MPa (Figure 2). Estimates of water volume from functional vessels  
174 combined with volume from non-functional vessels gave the total water content in vessels of  
175 transpiring plants across the range of experienced water potentials. The volume estimate is  
176 higher than that predicted by the PLC curve as it also contains water from non-functional vessels.

177 Plants recovering from severe stress ( $\Psi < -2.0$  MPa) had very low volumes of water in non-  
178 functional vessels (below that predicted based on water potential), while plants recovering from  
179 moderate stress ( $-2.0 < \Psi < -1.20$  MPa) showed volumes in the range expected for current water  
180 potential (Figure 2).

181 The osmotic potentials of water collected from functional vessels over the range of plant  
182 stress from well watered to moderately stressed plants were very low (in most cases below 0.05  
183 MPa, Fig. 3A). In the case of sap collected from non-functional vessels from the same range of  
184 the stress, osmotic potential was higher, reaching 0.4 MPa in plants stressed to -1.5 MPa (Fig.  
185 3B). Despite these much higher values, they were not close to a 1:1 relation with stress, i.e. the  
186 total osmotic potential of the liquid remaining in non-functional vessels could not balance the  
187 stress level. In liquid collected from functional vessels osmotic potential could be accounted for  
188 by the presence of cation based osmotica, as calculated from  $K^+$  equivalent concentrations.  
189 Sugars were almost not present in samples collected from stems with stress level above -1.5  
190 MPa, and only one sample, at -1.6 MPa, had an elevated sugar level. This chemical composition  
191 pattern dramatically changed in liquid collected from non-functional vessels. Here ion based  
192 osmotica constituted only 50% of total osmotica while the rest came from total soluble  
193 carbohydrates (estimated from the glucose concentration equivalent). Interestingly, the 50% level  
194 for sugar held over the entire range of water stress tested (Figure 3B).

195 The osmotic potential of liquid collected from functional vessels in plants recovering  
196 from stress was very similar to that of non-stressed plants (Figure 4). Average osmotic potential  
197 was  $\sim 0.03$  MPa, and could be accounted for by ions with very little contribution from sugars.  
198 Liquid collected from non-functional vessels had significantly higher osmotic potential (on  
199 average  $\sim 0.2$  MPa) than the liquid collected from functional vessels (t-Student p-value $<0.001$   
200 and DF=22, t=6.19), as well as a higher contribution from sugar based osmotica ( $\sim 50\%$ ). It is  
201 also important to note that the osmotic potential of sap from non-functional vessels in plants  
202 recovering from stress fell on a 1:1 ratio to balancing pressure (i.e., plant water potential).

203 Xylem sap pH collected from functional vessels of well-watered to moderately stressed  
204 plants ( $-1.2 < \Psi < -0.3$  MPa) was not correlated with the stress level and ranged between 5.5 and  
205 7.5. Severe water stress resulted in sudden drop of xylem sap pH to approximately 3.5. Such  
206 response was well described by a 'dose response curve' (Figure 5). Values of pH in non-  
207 functional vessels were significantly lower (pH = 5.44) than in functional vessels (pH = 6.18) for

208 the same interval of stem water potential (well watered and moderately stressed plant;  $-1.2 < \Psi <$   
209  $0.2$  MPa) (t-Student p-value $<0.001$  and DF=30,  $t=4.08$ ). However, there were no significant  
210 differences in the pH values of sap collected from the non-functional (pH = 5.82) and functional  
211 vessels (pH= 6.04) of plants recovering from stress (Figure 5).

212

## 213 **Discussion**

214

215 The research presented in this report provides a “first look” into the basic chemistry of  
216 refilling. Direct observations of water droplets in embolized vessels using cryo-SEM (Facette et  
217 al., 2001; Melcher et al., 2001) or observations of the dynamics of refilling process using X-ray  
218 microscopy (Lee and Kim, 2008; Brodersen et al., 2010), together with the analysis of xylem  
219 carbohydrate metabolism dynamics (Ameglio et al., 2004; Gupta and Kaur, 2005; Salleo et al.,  
220 2009; Secchi and Zwieniecki, 2011) and transcriptome activity of parenchyma cells (Secchi et  
221 al., 2011), provide indirect evidences that xylem parenchyma cells supply both the energy and  
222 water required to drive the refilling process (Zwieniecki and Holbrook, 2009). Further progress  
223 in our understanding refilling could come from linking observations of droplets dynamics with  
224 cellular activity, work that requires the ability to study the properties of water collected from  
225 embolized vessels, as demonstrated by a novel method used in this work. The results presented  
226 here are focused on the determination of the major components of the driving force to achieve  
227 refilling, and on the corresponding changes in the volume of water in non-functional vessels  
228 during the onset of stress. The pattern of drought induced embolism, and the ability to refill  
229 embolized vessels in *P. nigra*, was not qualitatively different from observations made on other  
230 species showing this behavior (Zwieniecki and Holbrook, 1998; Stiller and Sperry, 2002; Stiller  
231 et al., 2005; Lovisolo et al., 2008), even if this particular poplar specie showed a relatively high  
232 native level of embolism. It is possible that fast growing *P. nigra* utilizes only outer layers of  
233 vessels in similar way to *A. saccharum* (Melcher et al., 2003), while determination of PLC on  
234 short sections includes pool of permanently non-functional early wood vessels. Increased stress  
235 resulted in increased level of embolism, expressed here as percent loss of conductivity (PLC),  
236 while at any given stress level these plants maintained a particular PLC within a dynamic range,  
237 and, upon rehydration of the plants, recovery of xylem transport capacity was observed. Thus

238 the results for the sap compositions for *P. nigra* are likely to be generalizable to other species  
239 showing refilling under tension.

240 This analysis revealed several novel and important aspects of xylem refilling. The  
241 osmotic potential of liquid collected from non-functional vessels increased with increase of water  
242 stress in plant. However, it could not account for the total level of stress, leaving a significant  
243 gap in the free energy gradient required to move water from parenchyma cells into adjacent  
244 vessels, nor could this energy gap be explained by the <5% dilution of water from non-functional  
245 vessels with water from functional vessels (see contamination analysis in the method section).  
246 Yet, despite the observed energy gap there was a significant volume of water present in non-  
247 functional vessels, amounting up to 20% of total vessel volume. This relative volume was  
248 relatively constant until water stress levels became more negative than -1.2 MPa, when it  
249 suddenly dropped. The presence of water that is not under tension in stems of stressed plants,  
250 with osmotic water potential lower than that required to balance the water stress as estimated  
251 from the balancing pressure method, calls for future tests of the general assumption of free-  
252 energy equilibria across the plant stem. Theoretical considerations that suggest hydraulic  
253 isolation of xylem vessels is required for refilling (Vesala et al., 2003; Choat et al., 2009) may  
254 need to be further expanded to include temporal and spatial water potential disequilibria.  
255 Requirements for such hydraulic/energy isolated domains would underline the importance of  
256 stem transport sectoriality (Ellmore et al., 2006; Zanne et al., 2006), persistence of leaf traces,  
257 and the role of phylotaxy in protecting plants from embolism formation and allowing embolism  
258 repair against apparent energy gradient (Holbrook and Zwieniecki, 1999; Tyree et al., 1999;  
259 Zwieniecki and Holbrook, 2009). Temporal/spatial disequilibria of water potential in stems can  
260 also result from the hydraulic properties of xylem parenchyma cells if ratio of water volumes  
261 moving across them to resistance is relatively low.

262 The osmotic potential of liquid derived from non-functional vessels was more negative  
263 than that derived from functional vessel (approximately 5x lower for any given stress level). In  
264 addition, the osmotic potential of sap from functional vessels could be entirely accounted for by  
265 the measured concentrations of inorganic ions. In contrast, the osmotic potential of liquid from  
266 non-functional vessels came from an approximately 50/50 split between inorganic ions and sugar  
267 molecules. This increased concentration of sugars was already present in well watered plants,  
268 and the split remained similar through the entire range of plant water stress (from -0.2 to -1.5

269 MPa). The increased level of sugars in non-functional vessels persisted in plants that were  
270 recovering from moderate stress. In these plants sugars also accounted for ~50% of osmotic  
271 potential. These observations of increased concentration of sugars suggest that indeed  
272 parenchyma cells are involved in the sugar release to embolized vessels (Ameglio et al., 2004;  
273 Zwieniecki and Holbrook, 2009; Secchi et al., 2011; Secchi and Zwieniecki, 2011). These  
274 findings are also consistent with previous studies showing changes in starch content (Bucci et al.,  
275 2003; Salleo et al., 2004; Salleo et al., 2009; Nardini et al., 2011; Secchi and Zwieniecki, 2011)  
276 and transcriptome response to embolism in changes in sugar metabolism pathways (including  
277 starch degradation), (Secchi et al., 2011).

278         The increased sugar efflux to embolized vessels coincides with the efflux of inorganic  
279 ions, as in all samples from non-functional vessels ion concentrations were also elevated relative  
280 to sap from functional vessels. Previous study of transcriptome analysis have shown an increase  
281 in expression level of metal ion transporters in response to embolism, but no comparable  
282 increase in expression of sugar transporters (Secchi and Zwieniecki, 2011). However, membrane  
283 sucrose transporters are often bi-directional proton co-transporters, with the direction of transport  
284 depending on proton (pH) and sucrose concentration gradients across the plasma membrane. If  
285 indeed embolism presence triggers starch degradation, it will lead to increased symplastic  
286 sucrose concentration and stimulation of the sucrose efflux. Co-efflux of the protons would then  
287 lead to decrease of the apoplastic pH. Indeed, analysis revealed lower pH in the liquid collected  
288 from non-functional vessels (~5.4 pH) than in functional vessels (~6.2 pH). This drop in pH  
289 would eventually slow down the sucrose release, but it could be countered by the activity of  
290 metal ion anti-porters that would then generate an efflux of ions, and influx of protons, resulting  
291 in the maintenance of new pH homeostasis at a more acid apoplastic level. In addition efflux of  
292 protons related to sucrose outward transport could be counterbalanced with ATP-proton  
293 transporters. Under severe stress conditions apoplastic pH was very low (3.5). Such low  
294 apoplastic pH should strongly reduce the potential for efflux of sucrose from cells, preventing  
295 the buildup of sugar related osmoticum necessary to sustain the presence of water in non-  
296 functional vessels. Indeed, there was very little or no water collected from non-functional  
297 vessels at severe stress levels. The observed here levels of pH, sugar and ion concentrations in  
298 liquid collected from functional and non-functional across the plant water stress levels are  
299 consistent with known sucrose transporters properties and transcriptome activity.

300 In plants recovering from stress, the level of osmotica in non-functional vessels was on  
301 average adequate to account for the driving force required for refilling (i.e. counter balance the  
302 existing tension estimate; Figure 4), although the volume of water was not dramatically different  
303 than in plants never experiencing stress. Again the osmotica present differ between functional  
304 and non-functional vessels in the same way as in plants under stress, with large ~50%  
305 contribution of sugars to sap in non-functional vessels. Sap pH in recovering plants was only  
306 slightly lower in non-functional vessels (pH = 5.82) than in functional (pH = 6.04), but still  
307 consistent with the transport of sugars and ions. The slightly higher pH in non-functional vessels  
308 might reflect a reduction in refilling activity upon return to non-stress conditions. The fact that  
309 the osmotic potential of the liquid in non-functional vessels of recovering plants accounts for the  
310 free-energy required to refill is consistent with the notion that, in some plants, successful refilling  
311 requires reduction in the level of water stress (Hacke and Sperry, 2003). However, the fact that  
312 this concentration of osmotica is similar to that found in non-functional vessels currently at the  
313 stress they were recovering from underscores the possibility that hydraulic isolation allows for  
314 prolonged persistence of energy disequilibria between different vessels and parts of the stem.

## 315 316 **Conclusions**

317 This first look at the basic chemistry of liquid collected from non-functional vessels in  
318 combination with previously published studies provides new insight into the physiology of  
319 refilling (Figure 6). Embolism formation, or its presence, triggers a large set of transcriptome  
320 and physiological responses (Melcher et al., 2003; Arango-Velez et al., 2011; Nardini et al.,  
321 2011; Secchi et al., 2011; Perrone et al., 2012).

322 Transcription responses include up regulation of aquaporins, metal ion transporters, and  
323 carbohydrate metabolism but not sugar transporters (Secchi et al., 2011; Secchi and Zwieniecki,  
324 2011). Analyses of carbohydrate metabolism in multiple studies suggest degradation of starch to  
325 be associated with embolism formation (Salleo et al., 2009; Secchi and Zwieniecki, 2011). This  
326 presumably leads to sucrose accumulation in the cell that would trigger an efflux of sugar via  
327 sucrose proton co-transporters (Carpaneto et al., 2005; Sauer, 2007; Ayre, 2011; Geiger, 2011).  
328 Activity of sucrose co-transporters leads to an accumulation of sucrose and protons in the vessel  
329 walls/lumen. This study confirms the presence of sugars in non-functional vessels and their  
330 contribution to an osmotic driving force, as well as the presence of lower pH (suggesting efflux

331 of protons). Lower apoplastic pH might trigger activation of proton pumps and metal ion anti-  
332 porters (Secchi et al., 2011). The activity of the proton pumps and metal anti-porters can  
333 counteract the drop of pH due to sucrose efflux and stabilize it at desirable level. The generated  
334 ion efflux provides additional osmotica as shown in this report. Together sugars and ions can  
335 account for the driving force that could generate refilling process under low water stress (Figure  
336 4, during rehydration) or if embolized vessels are hydraulically isolated from functional xylem  
337 even during active transpiration under moderate stress (Figure 3). The interaction of sugar and  
338 metal ion channels that recirculate protons to accumulate osmoticum in the apoplast represents a  
339 physiological activity that would promote refilling, and that is strongly supported by the  
340 chemical properties of liquid from non-functional vessels found in this study (Figure 6).

## 341 342 **Materials and methods**

### 343 344 *Plant materials and experimental design*

345 *Populus nigra* cuttings were rooted into moist potting mix in a 5.7 x 8.3 cm Rose Pot.  
346 Plants were then transferred into 1 gallon pots filled with potting mix and were grown in a  
347 greenhouse for ten months (July – April). Ambient conditions in the greenhouse were  
348 characterized by a temperature maintained in the range 17 °C to 29 °C and the natural daylight  
349 was supplemented with light from metal halogen lamps (500-600  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) to  
350 maintain 12/12 hours of light/night cycle. Plants were approximately 1.2 to 1.5 m tall at the onset  
351 of the experiments.

352 A total of 53 *P. nigra* plants were used in this study, of these 10 plants were kept as  
353 controls. Control plants were watered to field capacity twice during the day (around 8 am and 2  
354 pm). Water stress was imposed in succession on the remaining 43 plants by a reduction of  
355 irrigation. Level of water stress was depending on drought duration. Approximately half of the  
356 control and stressed plants were used to collect xylem sap from functional vessels and to  
357 determine the level of loss of hydraulic conductivity (PLC). The remaining plants (both control  
358 and stressed) were used to collect xylem sap from non-functional vessels (details of the applied  
359 technique described below). All physiological measurements were performed in the morning  
360 from 9 am to 12 pm.



361 An additional 26 plants were first stressed and then rewatered in the morning (9 am) and  
362 the dynamics of refilling were followed. Measurements on plants recovering from stress were  
363 performed two hours after irrigation, with one final measurement implemented the following day  
364 at 9 am.

365

#### 366 *Measurements of stem water potential and stem hydraulic conductivity*

367 Stem water potential was measured for each plant using equilibrated non-transpiring  
368 (bagged) leaves. Mature leaves were covered with aluminum foil and placed in a humidified  
369 plastic bag for at least 15 minutes prior to excision and measurement. Fifteen minutes was shown  
370 an adequate time for hydraulic equilibration of non-transpiring leaves with stem water potential  
371 in some woody species (Fulton et al., 2001). In addition we tested the validity of the 15 minute  
372 equilibration time for our *P. nigra* plants (see Figure S1). After excision, leaves were allowed to  
373 equilibrate for a few minutes and water potential was measured using a Scholander-type pressure  
374 chamber (Soil Moisture Equipment Corp., Santa Barbara, CA, USA).

375 Following the determination of stem water potential, stem hydraulic conductivity was  
376 measured for half plants using a standard approach described previously (Secchi and Zwieniecki,  
377 2010). Briefly, small sections of stems (~4cm long) were cut under water to prevent embolisms  
378 caused by air entering into the cut vessels. The initial hydraulic conductance ( $k_i$ ) of each stem  
379 segment was measured by determination of the flow rate of a filtered 10 mM KCl solution  
380 through the stem section from a water source located on a balance (Sartorius  $\pm 0.01$  mg) and  
381 connected to the stem by a plastic tube. The stem was submerged in a water bath with the water  
382 level being ~10cm below the level of water on the balance. After a steady flow rate was reached  
383 (within just a few minutes), the tube connecting the stem to the balance was closed, and a bypass  
384 used to push water across the segment under ~ 2 bars of pressure for approximately 20 seconds  
385 to remove embolism. Stem conductance was then re-measured to find maximum conductance  
386 ( $k_{max}$ ). The percent loss of conductance (PLC) was calculated as  $PLC = 100 * (k_{max} - k_i) / k_{max}$ .

387

#### 388 *Xylem sap collection from functional vessels*

389 Xylem sap of functional vessels was collected from the same plants that were used to  
390 determine PLC values and stem water potential. A few seconds after cutting stem under water  
391 for PLC determination (described above), leaves were removed in order to prevent

392 evaporation and to avoid loss of water in functional vessels due to evaporation. A new cut 20  
393 cm above the first one was made (the 20 cm piece of stem, divided in three different sections,  
394 was used to determine PLC). The remaining whole stem was then attached through a plastic  
395 tube to a syringe needle. The needle was threaded through a rubber cork to a small vacuum  
396 chamber with needle tip placed in the 1.5 ml plastic tube. After generation of vacuum (0.027  
397 MPa absolute pressure), small pieces of stem were consecutively cut from the top allowing  
398 liquid from open vessels to be sucked out of the stem and collected in the tube. Collected  
399 liquid was then frozen and kept until further analysis. This method only allows for collecting  
400 liquid that near vacuum can remove from the stem (i.e., no liquid could be removed if it was  
401 separated from the applied suction by a bordered pit membrane).

402

#### 403 *Xylem sap collection from non-functional vessels*

404 A new approach was developed to collect liquid from non-functional vessels. Stems  
405 were cut in the air approximately 20 cm above the soil allowing for removal of water from  
406 functional conduits to the first border pit membrane field by apical plant suction (Fig. 7 step  
407 1). Plants were then placed in a plastic bag to prevent the development of further water stress.  
408 After this initial preparation, xylem sap was collected following these steps: stems were cut in  
409 air, allowing all non-embolized vessels to empty themselves due to preexisting suction within  
410 the remaining apical part of the plant. Water in functional vessels was presumably only  
411 sucked to the nearest (most basal) bordered pit membrane (Fig. 7 step 2). The first cut was  
412 then followed by second cut producing a ~4cm long stem section that could contain water in  
413 non-functional vessels (i.e. that was not under suction) and water from functional vessels that  
414 was held at the border pit field (Fig. 7 step 3). The ~4 cm stem segment was then rotated and  
415 the distal end placed into a tube connected to a small vacuum chamber (as described in the  
416 section above), while the proximal end was placed into a tube filled with low viscosity silicon  
417 oil (Fig. 7step 4 and 5). Application of vacuum forced oil to pass through all vessels that were  
418 open at both ends, removing any liquid from them, while vessels occluded by bordered pit  
419 fields would have remained impassible, as suction could not be translated through them thus  
420 allowing any remaining water from functional vessels to stay in the stem section (Fig. 7 step  
421 6). Since volumes of water collected with this method were very small, and could potentially  
422 evaporate in the vacuum environment, silicon oil was used to prevent that. Approximately 60-

423 80 cm of stem was consecutively cut to collect liquid from non-functional vessels. The oil  
424 filled tubes with suspended water droplets were then spun in a centrifuge to collect the water  
425 at the bottom, and its volume was measured. In order to estimate the total vessel volume (see  
426 below), the length and diameter of each stem section was determined.

427

428 *Test of the technique to collect water from non-functional vessels.*

429 The above technique for the collection of water from non-functional vessels was tested  
430 on a separate set of plants. A low concentration of sulforhodamine 101 dye was prepared  
431 such that it did not saturate the reading of light absorbance in 540nm wave length (Multiscan,  
432 Thermo Scientific). Then a calibration curve was made with a series of subsequent dilutions  
433 of the dye. This low concentration dye was then used to perfuse stems (1m long) of well  
434 watered plants (approximately -0.3 MPa water potential) using suction. Suction was applied  
435 as long as it was needed for dye to perfuse the whole stem, such that the collected dye was at  
436 the same level of absorption as the dye applied to the stem. Perfused stems were then cut in  
437 half and one section was used to collect water from functional vessels (see above), and the  
438 other section was used to collect water from non-functional vessels (see above).

439 Relative absorption of 100% was assigned to the initial dye. Liquid collected using  
440 functional vessels collection technique showed no dilution (101.9% with SD=4.67 and n=3).  
441 Liquid collected using non-functional vessel technique showed low level presence of the dye  
442 suggesting small contamination from functional vessels (3.8% with SD=7.05 and n=3). This  
443 small dilution was not significantly different from zero and thus it was not used to adjust  
444 determination of sap chemistry.

445

446 *Determination of vessel volume*

447 The PLC technique provides information about the percent of non-functional vessels,  
448 while liquid collected from non-functional vessels informed us about the volume of the water  
449 in stem that was not under tension at the time of collection. To allow for the comparative  
450 analysis of these two sets of data we conducted an analysis of stem segment vessel volume.  
451 Short pieces of stem segments (without bark) were perfused (2 bars pressure) with water.  
452 Then water was sucked from stem segments into 1.5 ml plastic tube using the system  
453 described above. Water volume was then determined and the stem's length and diameter were

454 measured. These measurements allowed for determination of the relationship between stem  
455 size and vessel volume, for subsequent assessment by non-functional water volume as a  
456 fraction of total vessel volume. An exponential function in the form of  
457  $V_v = 28.049 * e^{(0.001049 * S_v)}$  was fitted to the experimental data and later use to estimate total  
458 vessel volume ( $R^2 = 0.78$ , with  $p < 0.0001$ ), where  $V_v$  – vessel volume and  $S_v$  – stem volume.

459

#### 460 *Isopiestic determination of sap osmotic potential*

461 Xylem sap osmotic potentials were measured using an isopiestic psychrometer  
462 (Isopiestic Co., Lewes, MD, USA). The psychrometer vapor chambers were prepared with filter  
463 paper discs saturated with distilled water ultrafiltered to 18.02 Mohms (EMD Millipore,  
464 Billerica, MA USA). Osmotic potential of the sap was determined by placing a 5 to 10  $\mu$ l of  
465 sample on a thermocouple suspended in the vapor chamber, followed by measurement of the  
466 resulting voltage output. The osmotic potential corresponding to the measured output was found  
467 by linear interpolation between two voltages induced by known solutions that bracketed the  
468 voltage induced by the sample (Boyer 1995). The sequence of measurements was as follows.  
469 First, the ‘high’ known potential output reading was established, using 5 to 10  $\mu$ l of distilled  
470 ultrafiltered water ( $\psi = 0$ ) on the thermocouple. Next, the unknown sample output was recorded,  
471 followed by a known solution expected to have a lower (more negative) osmotic potential than  
472 the unknown, with that expectation based on the observation of the difference in output between  
473 the unknown sample and the first known solution. All three droplet measurements were made  
474 with the same thermocouple and vapor chamber. After the voltage output for the second known  
475 potential solution was recorded, the final estimate of the osmotic potential of the unknown was  
476 calculated as:  $\Psi_{sap} = \Psi_h - (V_h - V_{sap}) * (\Psi_h - \Psi_l) / (V_h - V_l)$ . Where:  $\Psi_{sap}$  = osmotic potential of the  
477 unknown sample;  $\Psi_h$  = osmotic potential of the low output solution (here 0);  $\Psi_l$  = osmotic  
478 potential of the known solution more negative than the unknown;  $V_{sap}$  = thermocouple voltage  
479 output generated by the unknown sample;  $V_{ch}$  = thermocouple voltage output generated by  
480  $\Psi_h$ ;  $V_{cl}$  = thermocouple voltage output generated by  $\Psi_l$ .

481

#### 482 *Carbohydrate content in xylem sap*

483 The anthrone-sulfuric acid assay described by (Leyva et al., 2008) was used to quantify  
484 the carbohydrate content in xylem sap samples. The anthrone reagent was prepared right before

485 analysis by dissolving 0.1 g of anthrone (0.1%) in 100 mL of concentrated sulfuric acid (98%).  
486 Standard solutions were prepared diluting a Glucose Standard Solution (1.0 mg/ml; Sigma, Saint  
487 Louis, Missouri, USA).

488 Briefly, 150  $\mu$ l of anthrone reagent were added to each well of the microplate containing  
489 50  $\mu$ L of standard solutions, positive control (water), xylem sap solutions and blank. Plates were  
490 then kept 10 min at 4 °C. Then, the plates were incubated 20 min at 100 °C. After heating, plates  
491 were cooled for 20 min at room temperature and absorbance at 620 nm was read with a  
492 microplate multiscan reader (Thermo Scientific Multiskan FC, Vantaa, Finland). Colorimetric  
493 response was compared to the glucose standard curve (5 mM, 1.5mM, 0.5mM, 0.15 mM,  
494 0.05mM), and total carbohydrate content was calculated as mg/mL of glucose. From the deduced  
495 molal concentration of each xylem sap solution, the relative osmotic potential was calculated  
496 based on the law for perfect gases  $\Pi = miRT$ , where:  $m$  = molality of the solution (moles of  
497 solutes/1000g H<sub>2</sub>O);  $i$  = a constant that accounts for ionization of the solute, for glucose  $i = 1$ ;  $R$   
498 = the gas constant (0.00831 liter MPa mol<sup>-1</sup> K<sup>-1</sup>);  $T$  = temperature, 293.16 K.

499

#### 500 *Measurement of ion concentration and pH*

501 Electrical conductivity measurements of liquid samples were performed with a custom  
502 made system. A 5 $\mu$ l capillary was fitted with gold electrodes at the both ends and connected to a  
503 digital multi-meter (True RMS digital multi-meter 289, Fluke Europe B.V., Eindhoven,  
504 Netherlands ). Liquid samples were sucked into the capillary using a pipettor. After each  
505 measurement, the pipet was washed with DI water and air dried. Before and after each set of  
506 measurements, a series of potassium chloride solution with different concentrations was used to  
507 establish a new calibration curve. Thus electrical conductivity reflects the equivalent  
508 concentration of potassium ions.

509 The xylem sap from functional and non-functional vessels was collected in 100 mm<sup>3</sup>  
510 tubes and kept on ice before the pH of each sample was measured using a micro pH electrode  
511 (MicroElectrodes Inc, Bedford, NH, USA).

512

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516

517 **References**

518

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651  
652  
653

654 **Figure legends**

655

656 **Figure 1**

657 *P. nigra* percent loss of conductivity (PLC) in relation to stem water potential. Data were fitted  
658 with dose response curve (full line) in the form of:  $PLC = \min_{PLC} + (\max_{PLC} -$   
659  $\min_{PLC}) / (1 + (\Psi/EC50)^{\text{slope}})$ , where  $\min_{PLC}$  was minimum PLC in non-stressed plants (49.1%);  
660  $\max_{PLC}$  was 100%; EC50 represent 50% loss of initial functionality:  $\min_{PLC} + (\max_{PLC} - \min_{PLC}) / 2$ ,  
661 and slope the rate of PLC increase at EC50. Dashed line represents 95% confidence interval for  
662 the fit. PLC of plants recovering from stress was not used in the fitting procedure.

663

664 **Figure 2**

665 Analysis of water volume in functional and non-functional vessels of *P. nigra* in relation to water  
666 stress level.

667

668 **Figure 3**

669 Changes in osmotic potential of liquid collected from functional (A) and non-functional vessels  
670 (B) in relation to stem water potential (balancing pressure at the time of sample collection). Total  
671 osmotic potential  $\pi$  was determined using isopiestic psychrometric measurements. Estimation of  
672  $\pi$  from sugar was calculated as equivalent of glucose content, and  $\pi$  from ions as equivalent of  
673  $K^+$  ion concentration. Dotted line represents 1 to 1 relation between balancing pressure and  
674 osmotic potential of liquid.

675

676 **Figure 4**

677 Osmotic potential of liquid collected from functional and non-functional vessels of plants  
678 recovering from moderate stress ( $-2.0 < \Psi < 1.0$  MPa). Total osmotic potential of liquid collected  
679 from non-functional vessels was significantly higher than that collected from functional vessels  
680 (t-Student  $p < 0.001$ ). Composition of the osmoticum also differed between two water sources  
681 with ions being major component in functional vessels and equal importance of sugars and ions  
682 in non-functional vessels.

683

684 **Figure 5**

685 Relationship between xylem sap pH and stem water potential. Sap from functional vessels was  
686 fitted with 'dose response curve':  $pH = \min_{pH} + (\max_{pH} - \min_{pH}) / (1 + (\Psi/EC50)^{\text{slope}})$  with  
687 parameters being:  $\max_{pH} = 6.26$ ,  $\min_{pH} = 3.42$ ,  $EC50 = -1.43$  and  $\text{slope} = 10$  ( $R^2 = 0.71$ ). Volume of  
688 liquid collected from non-functional vessels of severely stressed plants was not enough to  
689 measure pH. No obvious relationship between pH and plant water stress was found for liquid  
690 from non-functional vessels from linear fit ( $R^2 = 0.01$ ).

691

### 692 **Figure 6**

693 Schematic illustration of membrane level physiology of refilling. See text for explanation. Red  
694 arrows represent fluxes. Blue arrows represent action/influence. Green stars represent  
695 information available from the former studies. Red stars represent new information from  
696 presented analysis.

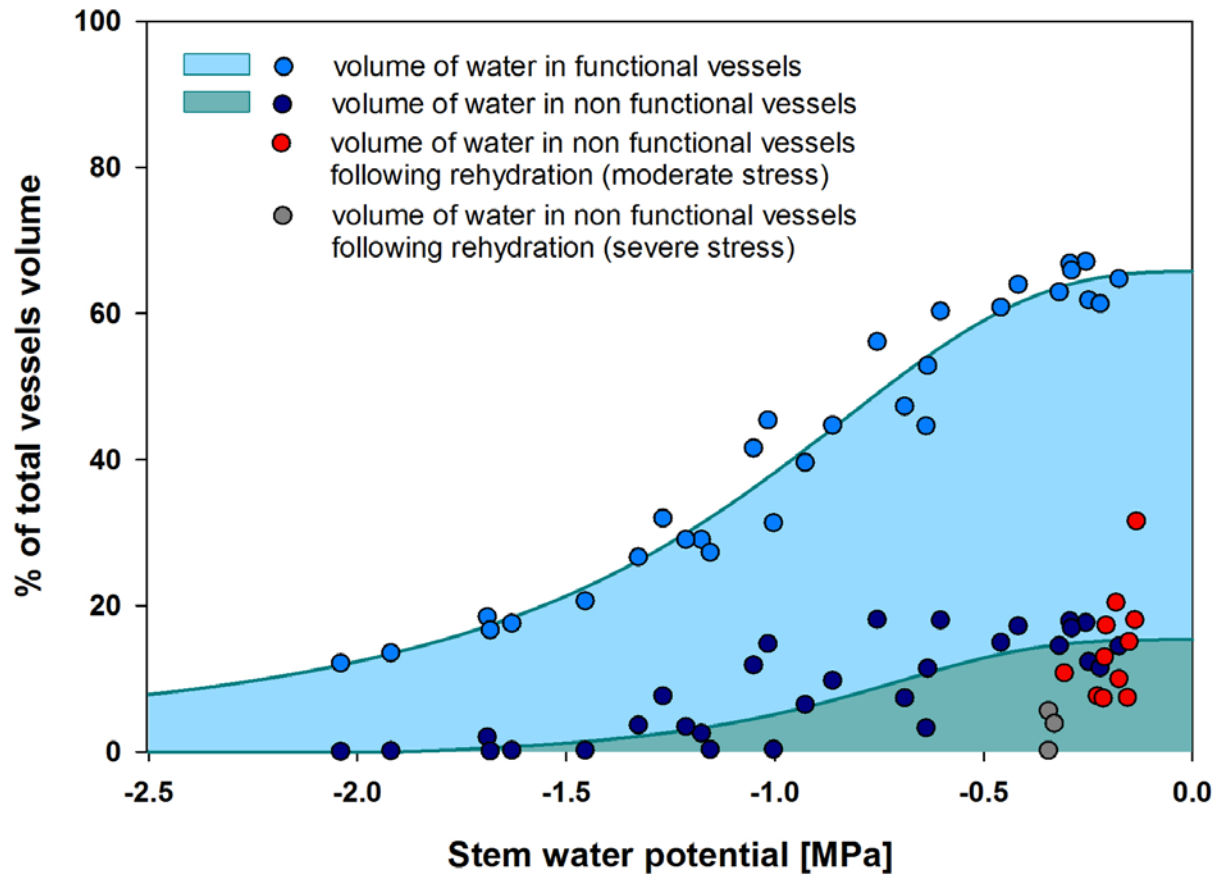
697

### 698 **Figure 7**

699 Schematics present technical steps involved in collecting sap from non-functional vessels. (1)  
700 Represent intact plant with (a) and (c) representing functional vessels under tension and (b) non-  
701 functional vessel partially filled with water. (2) Collection starts with first cut made in the air.  
702 This would make water under tension to be sucked toward the leaves in both (a) and (c) but not  
703 water present in (b). In (c) vessel water would be only sucked to the nearest border pit field. (3)  
704 Within several seconds following the first cut a second cut is made and a portion of stem (3-4  
705 cm) long is removed. It presumably contains some water under tension stack on the bordered pit  
706 field (c) and water in non-functional vessels. (4) Section is then inverted and both ends fitted to  
707 flexible tubes. (5) Upper tube is then filled with low viscosity silicon oil and lower end fitted to  
708 vacuum system. (6) Vacuum is generated that sucks oil through the empty vessels (a), and  
709 vessels that are open across the stem but filled with water droplets (b). However, vacuum is not  
710 adequate to break water away from the border pit field (c). Oil containing small volumes of  
711 water from non-functional vessels is collected in centrifuge tubes and protect small droplets from  
712 evaporation in vacuum conditions. After several collection cycles centrifuge tubes are being  
713 spun and water is separated from oil at the bottom of the tube. Arrows and flat ended lines  
714 represent movement of water in vessel during procedure of water collection.

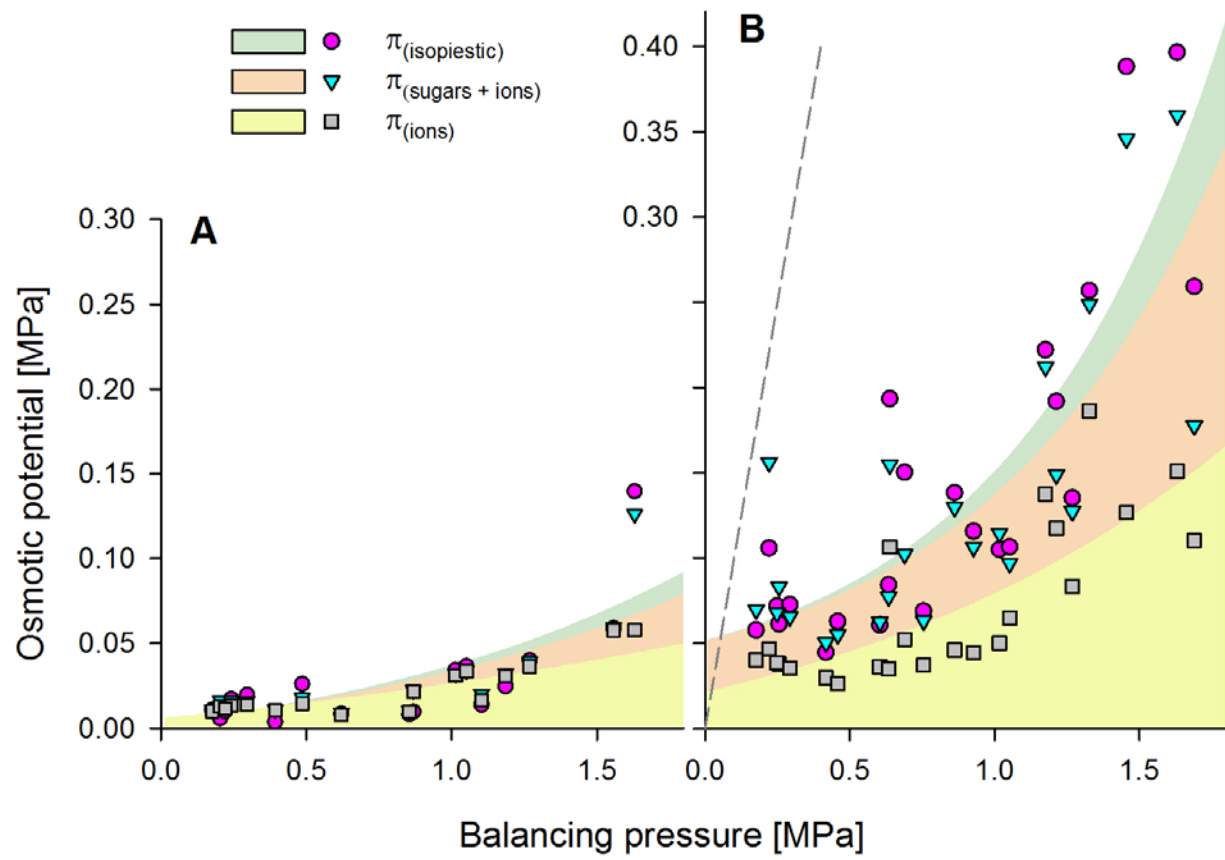
715





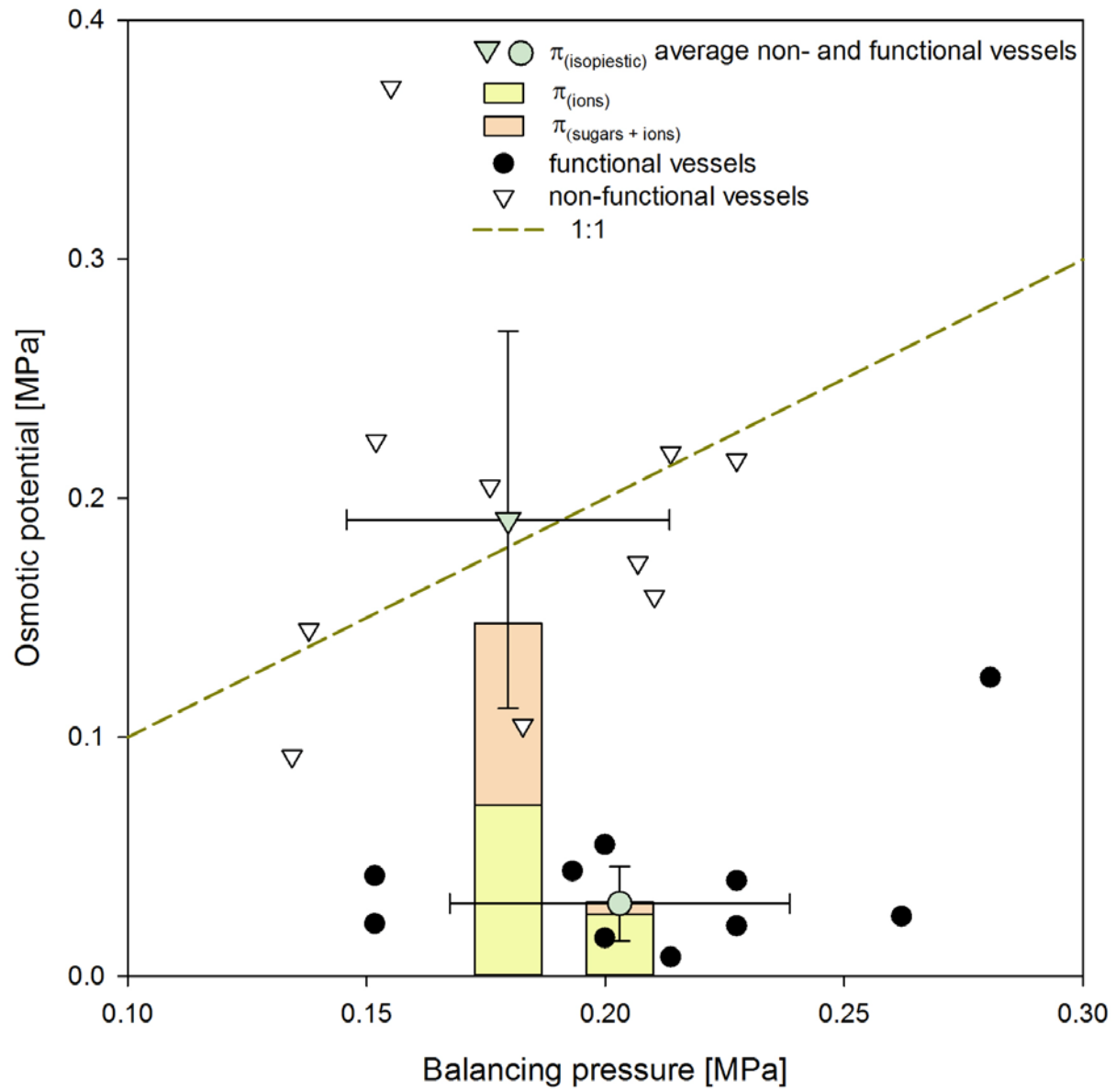
719

720 **Figure 3**



721

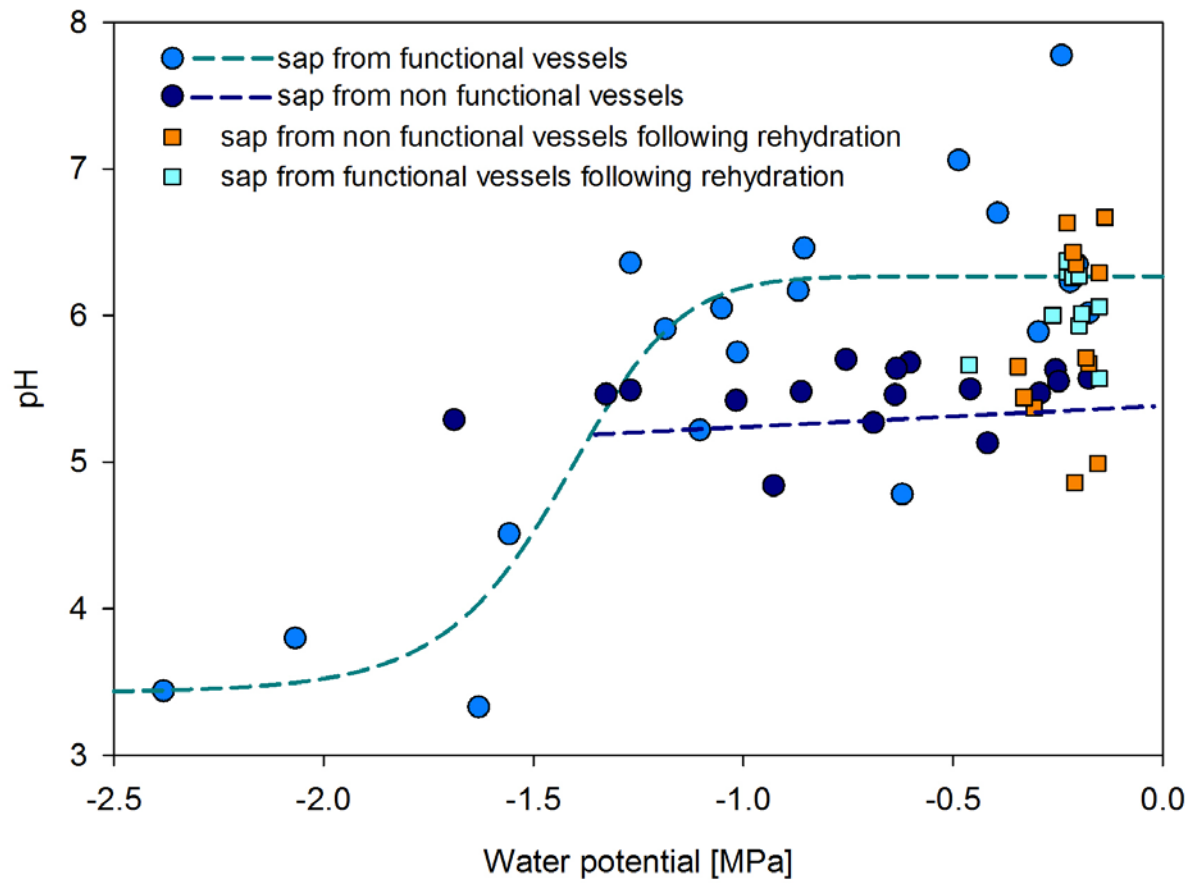
722 **Figure 4**



723

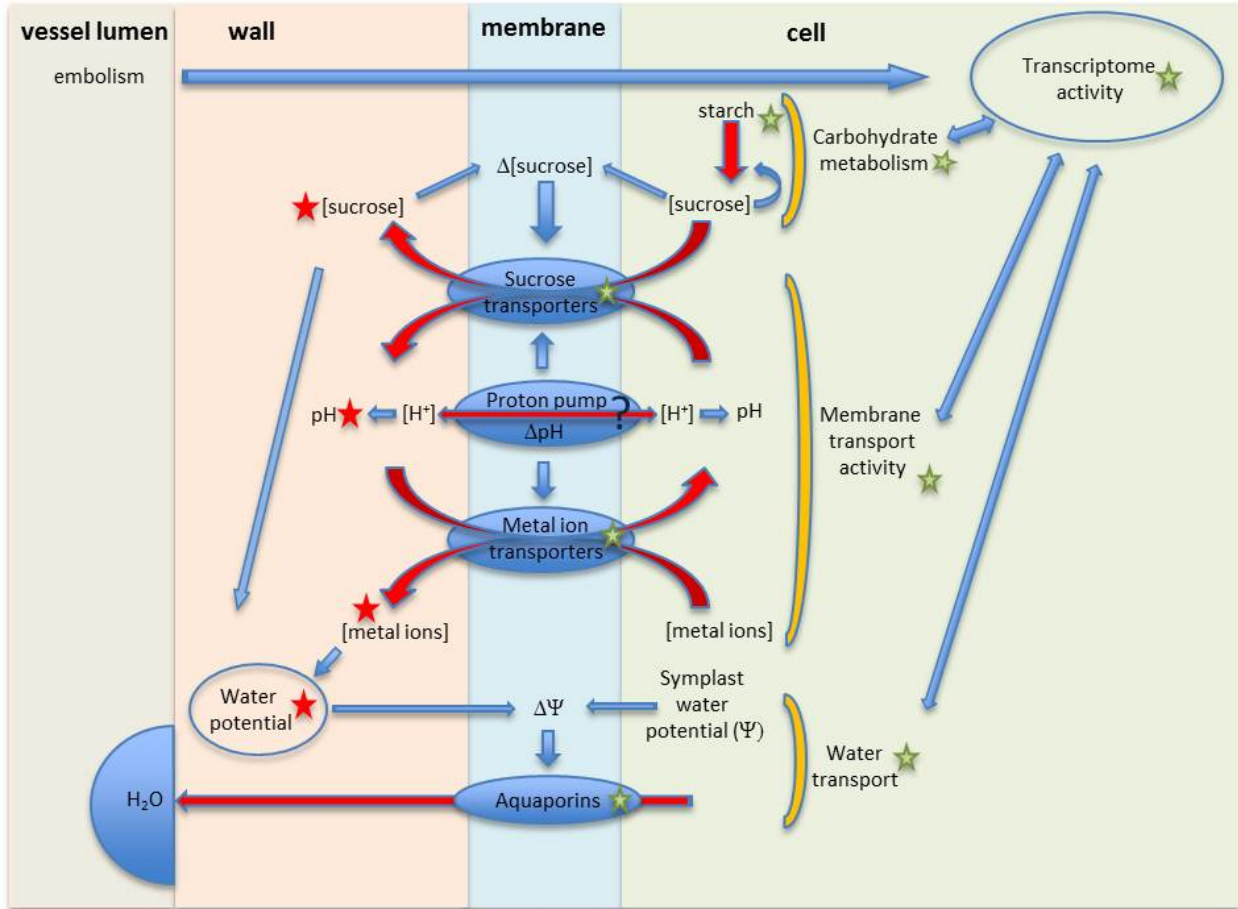
724 **Figure 5**





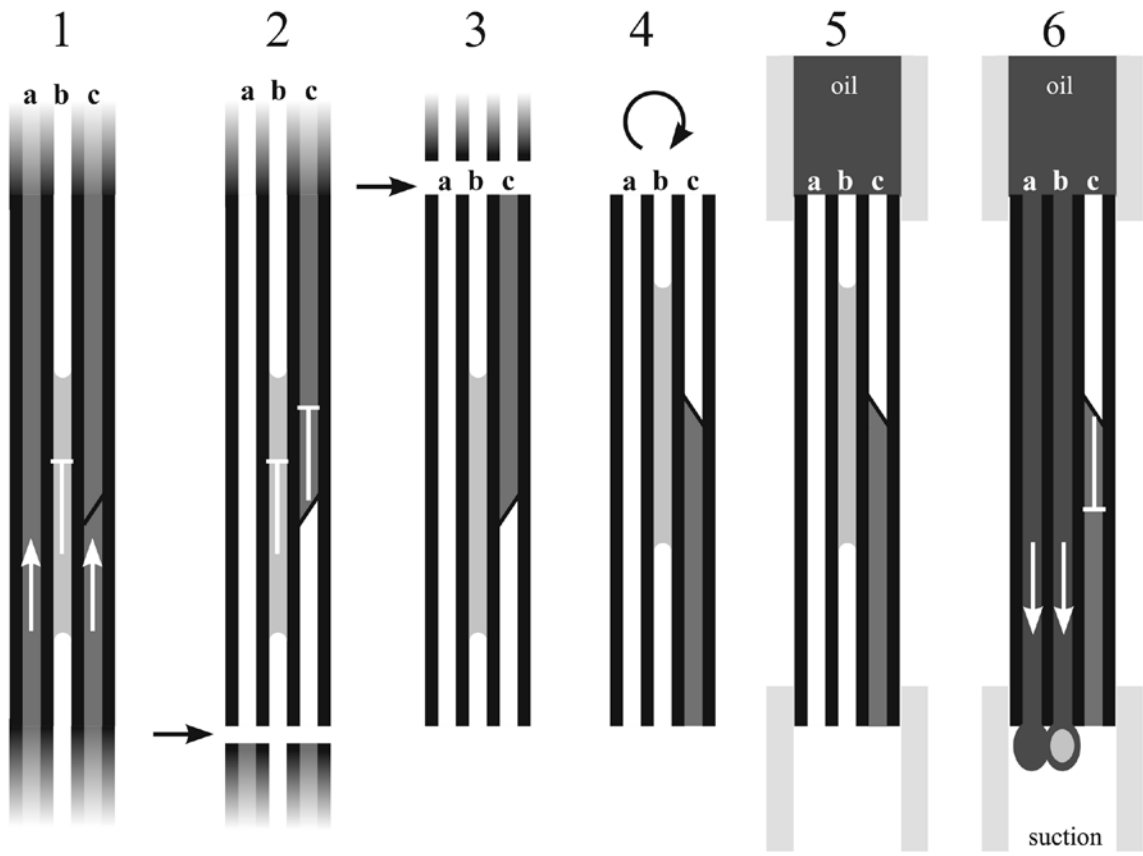
725

726 **Figure 6**



727

728 **Figure 7**



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730