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**Chemistry of xylem embolism refilling.**

Corresponding author:
Francesca Secchi,
Arnold Arboretum of Harvard University,
1300 Centre Street.
Boston, MA 02131
USA
617 3845182
Email: fsecchi@oeb.harvard.edu

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

Francesca Secchi[1], and Maciej A Zwieniecki[1,2]

[1] Arnold Arboretum of Harvard University, Boston, MA, USA;

[2] Department of Plant Sciences, UC Davis, Davis, CA, USA

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Corresponding author:
Francesca Secchi
Email: fsecchi@oeb.harvard.edu

**Abstract**

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

## Introduction

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

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

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

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

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

## Results

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

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

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

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

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

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

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

## Discussion

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

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

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

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

## Conclusions

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

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

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

## Materials and methods

*Plant materials and experimental design*

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

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

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

*Measurements of stem water potential and stem hydraulic conductivity*

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

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

*Xylem sap collection from functional vessels*

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

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

*Xylem sap collection from non-functional vessels*

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

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

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

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

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

*Determination of vessel volume*

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

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

*Isopiestic determination of sap osmotic potential*

Xylem sap osmotic potentials were measured using an isopiestic psychrometer (Isopiestics Co., Lewes, MD, USA]. The psychrometer vapor chambers were prepared with filter paper discs saturated with distilled water ultrafiltered to 18.02 Mohms (EMD Millipore, Billerica, MA USA). Osmotic potential of the sap was determined by placing a 5 to 10 µl of sample on a thermocouple suspended in the vapor chamber, followed by measurement of the resulting voltage output. The osmotic potential corresponding to the measured output was found by linear interpolation between two voltages induced by known solutions that bracketed the voltage induced by the sample (Boyer 1995). The sequence of measurements was as follows. First, the 'high' known potential output reading was established, using 5 to 10 µl of distilled ultrafiltered water ($\psi=0$) on the thermocouple. Next, the unknown sample output was recorded, followed by a known solution expected to have a lower (more negative) osmotic potential than the unknown, with that expectation based on the observation of the difference in output between the unknown sample and the first known solution. All three droplet measurements were made with the same thermocouple and vapor chamber. After the voltage output for the second known potential solution was recorded, the final estimate of the osmotic potential of the unknown was 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 unknown sample; $\Psi_h$ = osmotic potential of the low output solution (here 0); $\Psi_l$ = osmotic potential of the known solution more negative than the unknown; $V_{sap}$ = thermocouple voltage output generated by the unknown sample; $Vc_h$ = thermocouple voltage output generated by $\Psi_h$;$Vc_l$ = thermocouple voltage output generated by $\Psi_l$.

*Carbohydrate content in xylem sap*

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

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

Briefly, 150 µl of anthrone reagent were added to each well of the microplate containing 50 µL of standard solutions, positive control (water), xylem sap solutions and blank. Plates were then kept 10 min at 4 °C. Then, the plates were incubated 20 min at 100 °C. After heating, plates were cooled for 20 min at room temperature and absorbance at 620 nm was read with a microplate multiscan reader (Thermo Scientific Multiskan FC, Vantaa, Finland). Colorimetric response was compared to the glucose standard curve (5 mM, 1.5mM, 0.5mM, 0.15 mM, 0.05mM), and total carbohydrate content was calculated as mg/mL of glucose. From the deduced molal concentration of each xylem sap solution, the relative osmotic potential was calculated based on the law for perfect gases $\Pi = miRT$, where: $m$ = molality of the solution (moles of solutes/1000g $H_20$); $i$ = a constant that accounts for ionization of the solute, for glucose $i = 1$; $R$ = the gas constant (0.00831 liter MPa $mol^{-1}$ $K^{-1}$); $T$ = temperature, 293.16 K.

*Measurement of ion concentration and pH*

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

The xylem sap from functional and non-functional vessels was collected in 100 mm3 tubes and kept on ice before the pH of each sample was measured using a micro pH electrode (MicroElectrodes Inc, Bedford, NH, USA).

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**Figure legends**

**Figure 1**

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

**Figure 2**

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

**Figure 3**

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

**Figure 4**

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

**Figure 5**

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

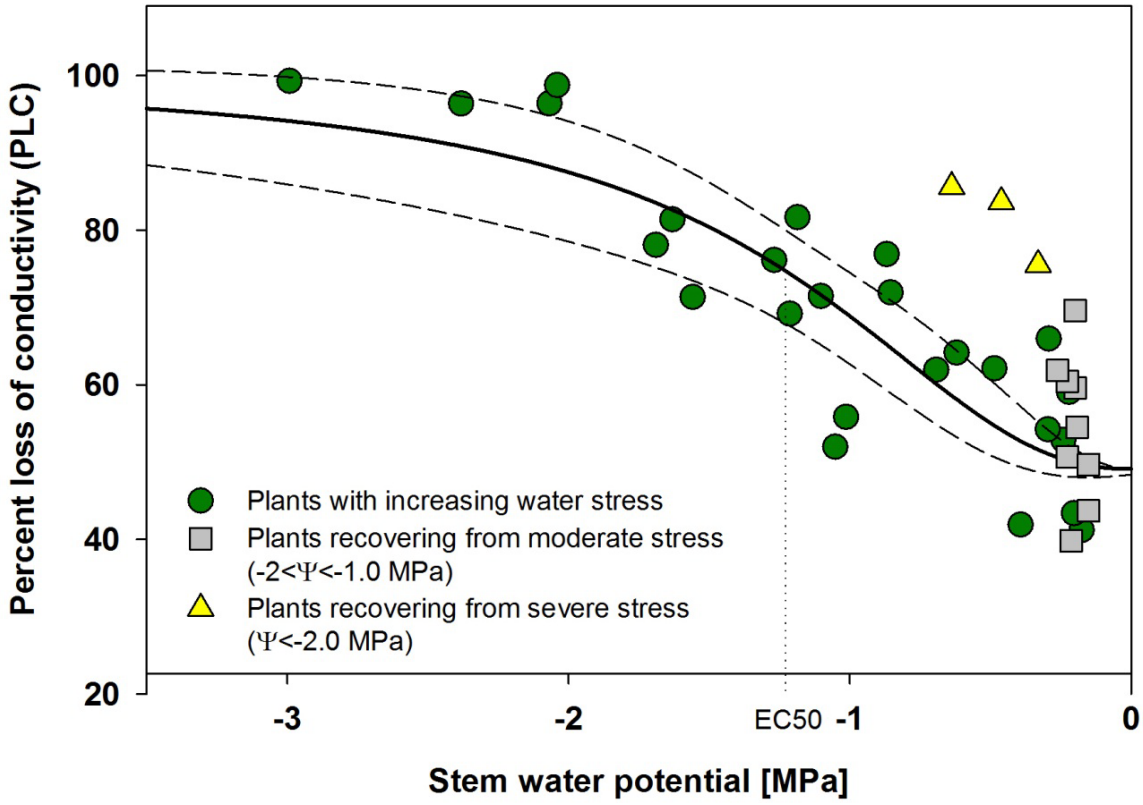
**Figure 6**

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

**Figure 7**

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

716 **Figure 1**

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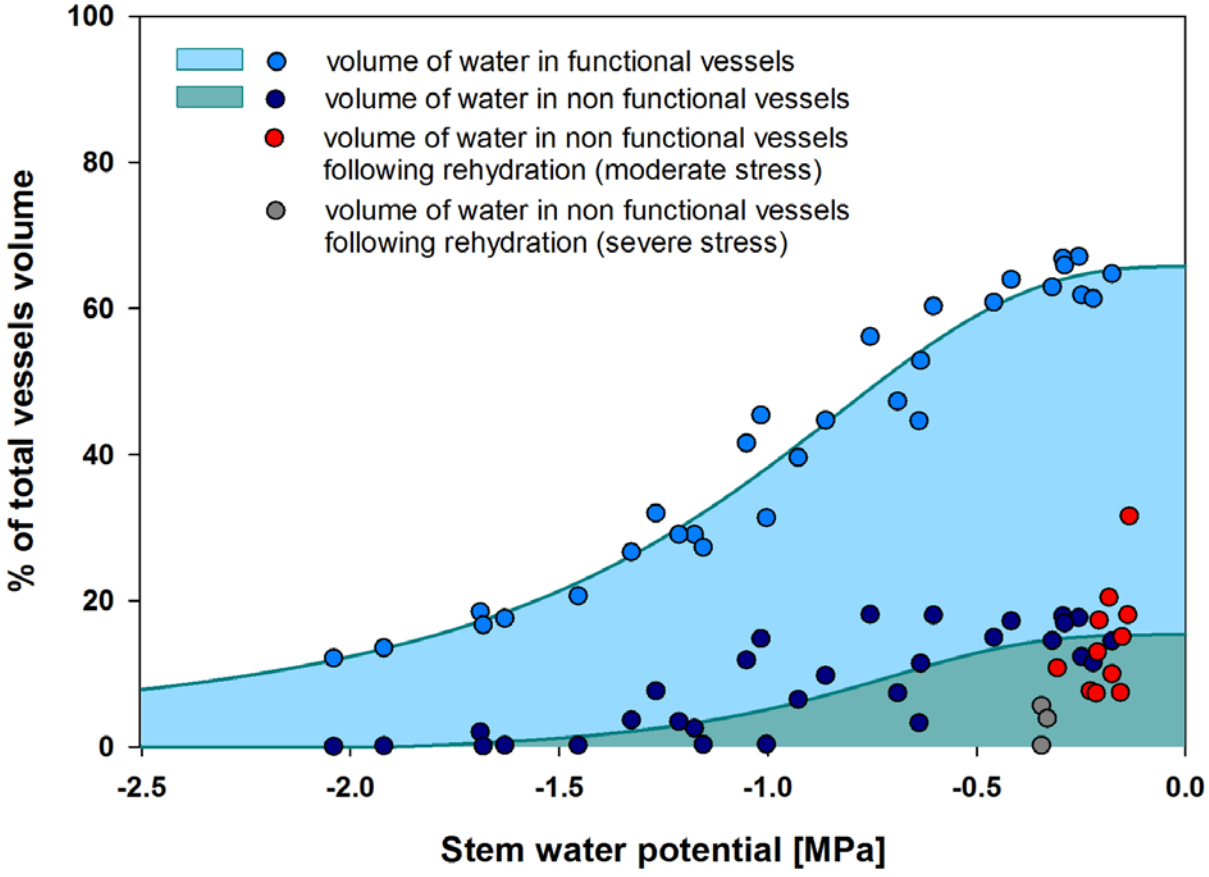


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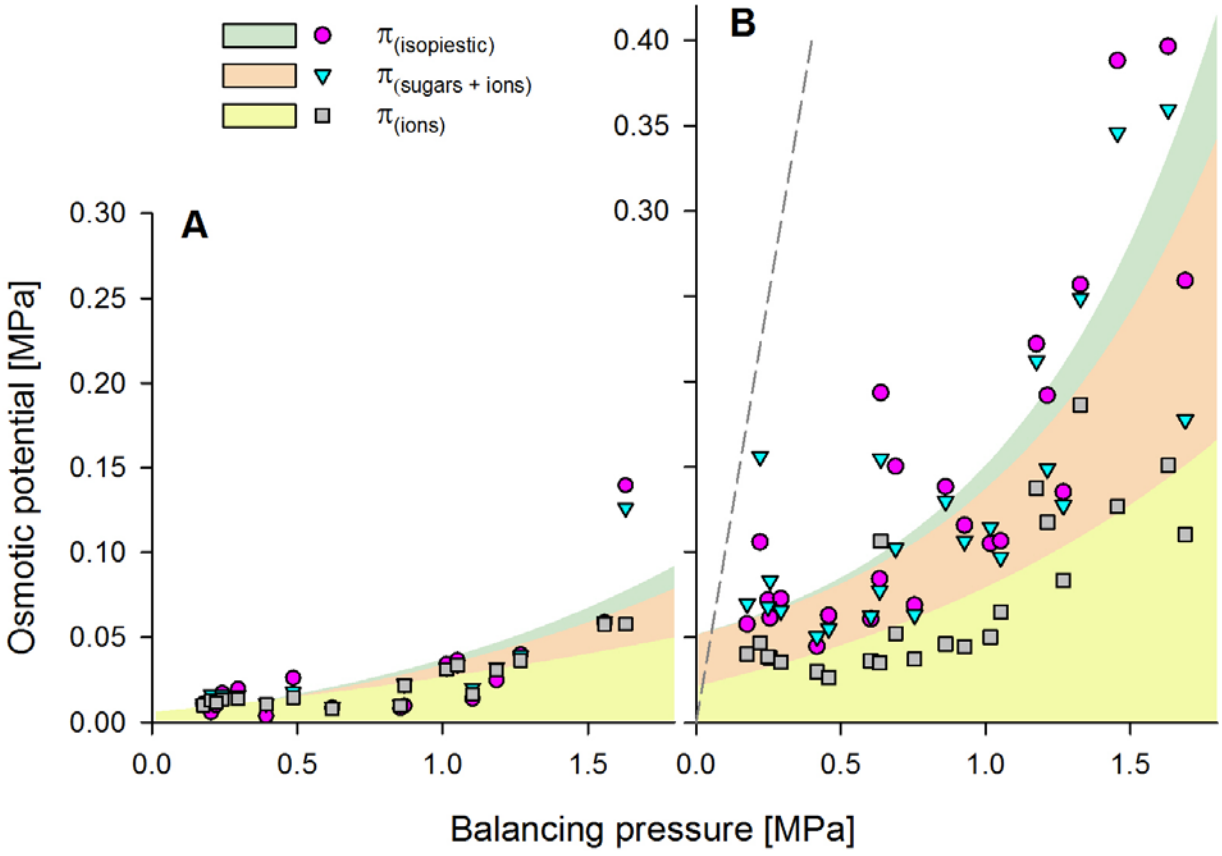


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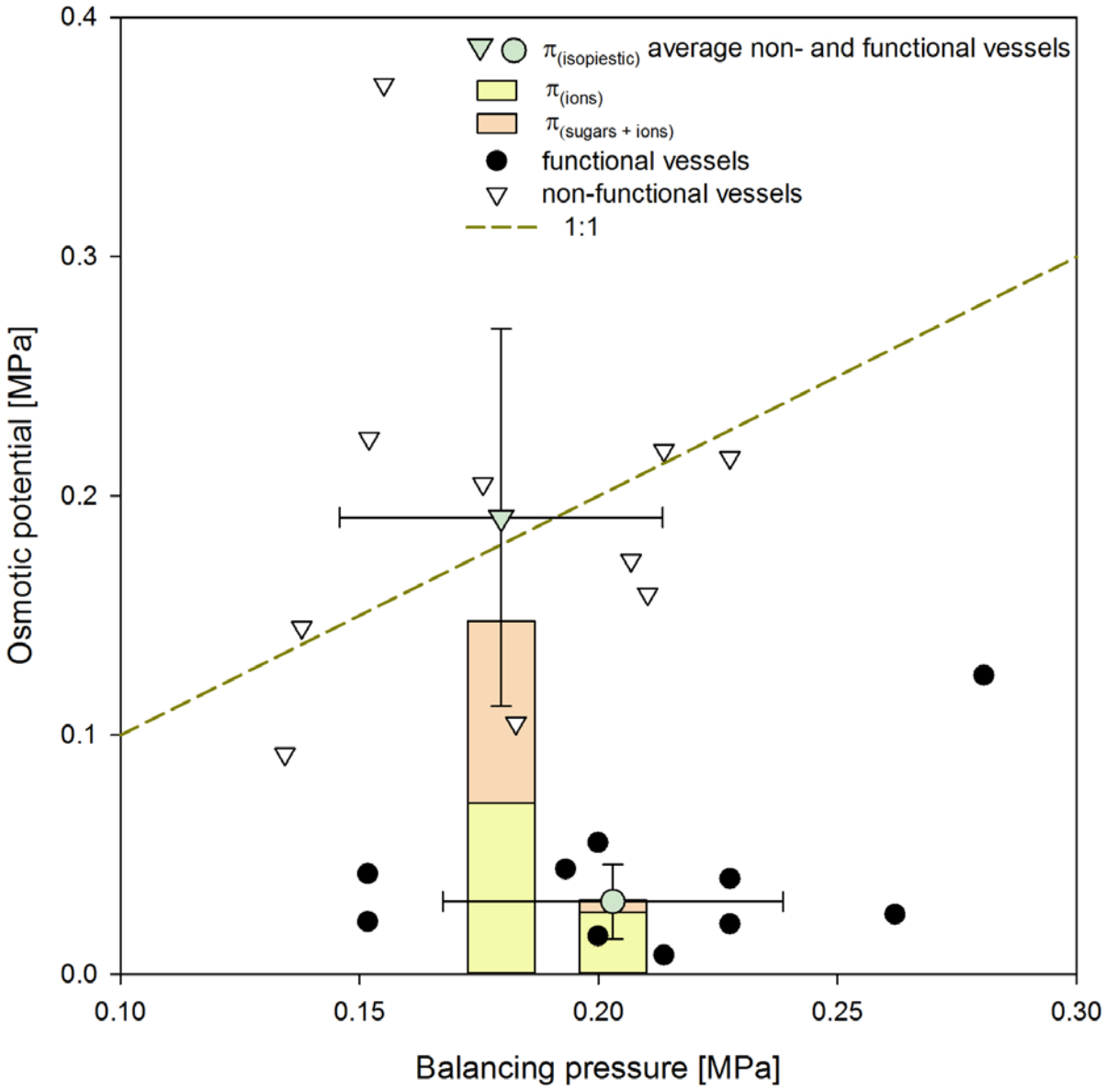


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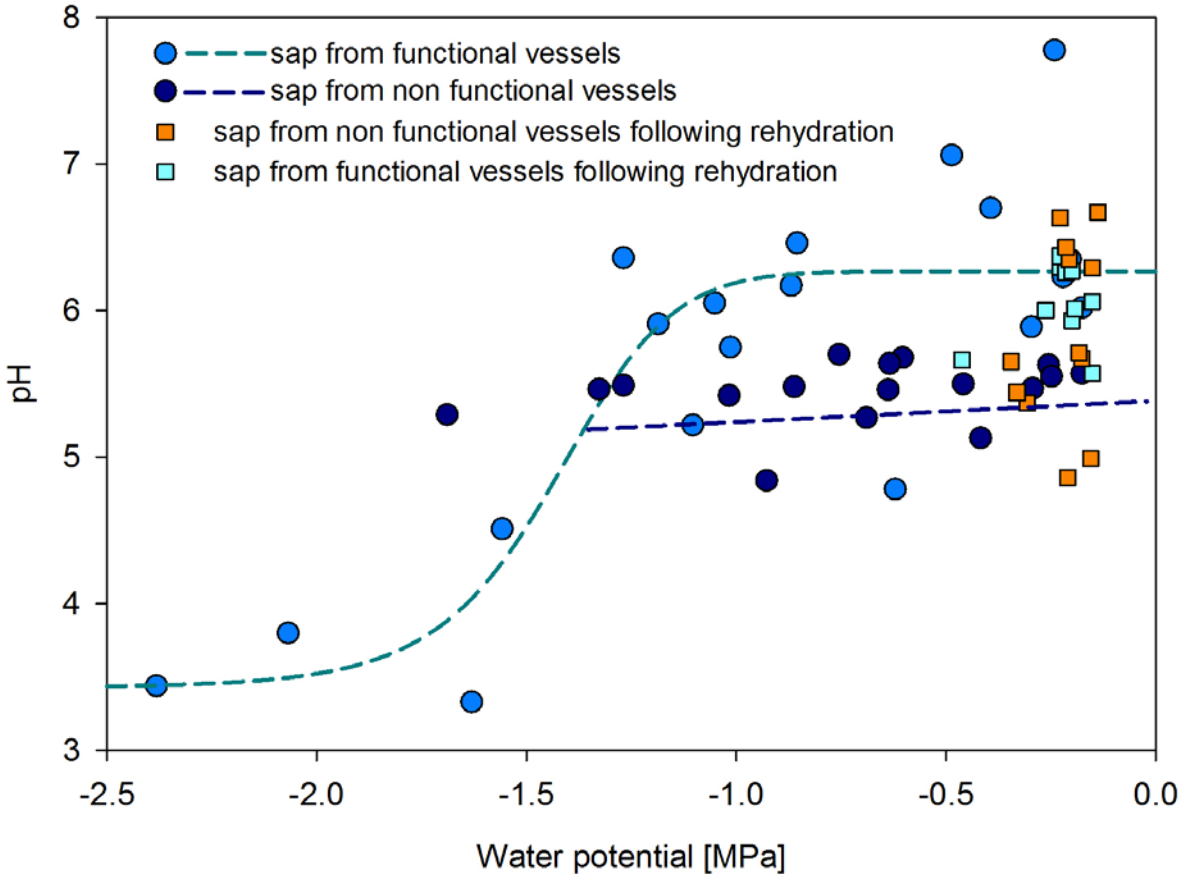


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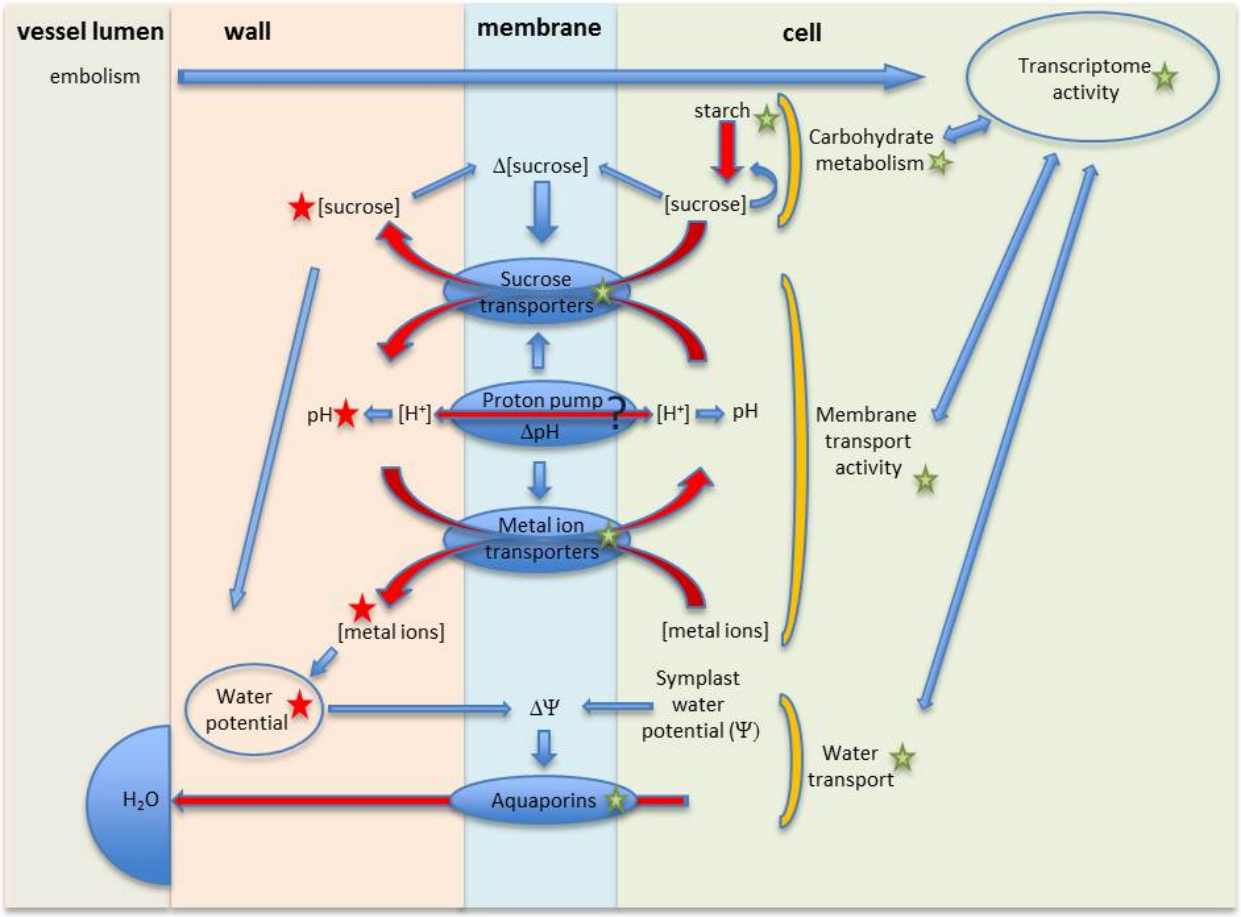


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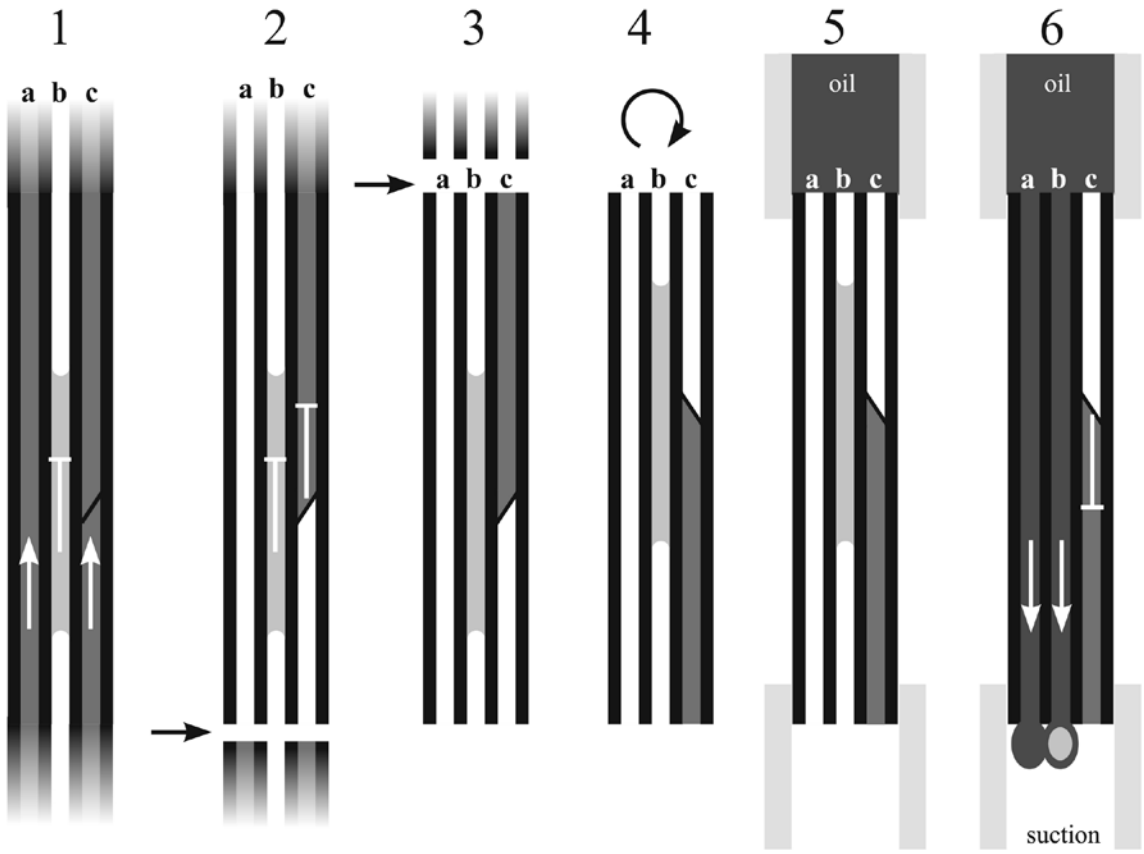


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