Title: Storage compartments for capillary water rarely refill in an intact woody plant

Authors: Thorsten Knipfer1*, Italo F. Cuneo2, J. Mason Earles1,3, Clarissa Reyes1, Craig R. Brodersen3, Andrew J. McElrone1,4*

Institution Addresses:
1Department of Viticulture and Enology, University of California, Davis, CA 95616. USA
2School of Agronomy, Pontificia Universidad Católica de Valparaíso, Quillota, Chile.
3School of Forestry and Environmental Studies, Yale University, New Haven, CT 06511, USA
4USDA-ARS, Crops Pathology and Genetics Research Unit, Davis, CA 95618, USA

Footnotes:
*CORRESPONDING AUTHORS; E-MAIL: tmknipfer@ucdavis.edu, ajmcelrone@ucdavis.edu,
T.K. designed and performed most of the experiments, analyzed the data, and wrote the article together with A.J.M.
I.F.C., J.M.E., C.R. performed some of the experiments, helped in data analysis, and revised the article.
C.R.B. helped in experimental design, performed the ESEM imaging, and revised the article.
A.J.M. obtained the grants, helped in experimental design, performed some of the experiments, and wrote the article together with T.K.

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Summary: Visualization of refilling dynamics in L. nobilis revealed that both capillary water storage and vessel refilling play a minor role under in-vivo conditions.

Running Head:
Visualization of water storage and refilling

Corresponding Authors:
Thorsten Knipfer
Department of Viticulture and Enology,
University of California,
Davis, CA 95616, USA
Phone: 530-752-1762
E-mail: tmknipfer@ucdavis.edu

Andrew J. McElrone
Department of Viticulture and Enology,
USDA-ARS, Crops Pathology and Genetics Research Unit,
University of California,
Davis, CA 95616, USA
Phone: 530-754-9763
Email: ajmcelrone@ucdavis.edu

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ABSTRACT
Water storage is thought to play an integral role in the maintenance of whole plant water balance. The contribution of both living and dead cells to water storage can be derived from rehydration and pressure-volume curves on excised plant material, but the underlying tissue-specific emptying/refilling dynamics remain unclear. Here, we used X-ray computed microtomography (microCT) to characterize refilling of xylem fibers, pith cells and vessels under both excised and *in-vivo* conditions in *Laurus nobilis*. In excised stems supplied with H_2O, water uptake exhibited a biphasic response curve, and microCT images showed that high water storage capacitance was associated with fiber and pith refilling as driven by capillary forces; fibers refilled more rapidly than pith cells while vessel refilling was minimal. In excised stems that were sealed, fiber and pith refilling was associated with vessel emptying, indicating a link between tissue connectivity and water storage. In contrast, refilling of fibers, pith cells and vessels was negligible in intact saplings over two timescales, a period of 24-h and 3-weeks. However, those compartments did refill slowly when the shoot was covered to prevent transpiration. Collectively, our data i) provide direct evidence that storage compartments for capillary water refill in excised stems but rarely under *in-vivo* conditions, ii) highlight that estimates of capacitance from excised samples should be interpreted with caution as certain storage compartments may not be utilized in the intact plant, and iii) question the paradigm that fibers play a substantial role in daily discharge/recharge of stem capacitance in an intact tree.

**Keywords:** embolism, fiber, hydraulic capacitance, *in-vivo*, parenchyma, pith, x-ray micro-tomography, xylem

**INTRODUCTION**
Stem internal water storage can prolong vessel functionality by sourcing water into the transpiration stream and reducing the risk of gas emboli by buffering xylem tension (Tyree and Sperry, 1989; Holbrook, and Sinclair, 1992; Holbrook, 1995; Cochard et al., 2013). Estimates of water storage are commonly derived from water-release curves measured on excised stems (Tyree and Yang, 1990; Jupa et al., 2016). These data indicate that water stored in dead fibers, non-functional vessels and apoplastic pores provide the largest fraction of stored water in most trees (i.e. capillary water storage). Living cells (e.g. xylem parenchyma) typically provide a relatively small storage volume due to their limited elasticity, and this water is released under more negative xylem pressures (i.e. elastic water storage). Experiments by Borchert and Pockman (2005) indicate that storage compartments for capillary and elastic water refill during stem rehydration, and refilling of capillary water storage requires xylem pressures of >-0.5 MPa. However, the hydraulic methods typically used to measure elastic and capillary water storage require excised plant material released from sustained negative pressures, and the resulting data does not provide detailed information about the temporal, spatial, or tissue-specific emptying or refilling dynamics of an intact plant.

Measurements on forest trees using sap flow sensors, isotopic tracers, frequency domain reflectometry, and dendrometers indicate that the emptying and refilling of storage compartments occurs on a daily basis (e.g. Goldstein et al., 1998; Cermak et al., 2007; Meinzer et al., 2009; Hao et al., 2013; De Schepper et al., 2012; Carrasco et al., 2014). For example, a release of stored water from the trunk can contribute up to 50% to daily transpiration (Waring et al., 1979; Verbeeck et al., 2007), and tree transpiration can be maintained with stored water for about one week (Cermak et al., 2007). Current data suggests that the volume of stored and discharged can be substantial, which implies that tissue compartments of relatively high storage capacity (such as dead fibers for storage of capillary water) are involved in this process and are able to refill on a daily basis. Similar to refilling of capillary water storage compartments (Borchert and Pockman, 2005), successful vessel refilling appears to require xylem pressures approaching or exceeding 0 MPa (Hacke and Sperry, 2003; Charrier et al., 2016). Therefore, both refilling processes may be interrelated, and vessel refilling in some species may be limited by a simultaneous recharge of capacitive tissue and competition for ‘free’ water. The link among refilling of water storage compartments and vessel refilling remains to be determined.

A large body of literature has been published over the last few decades concerning the importance of plant water storage (examples cited above), but the tissue compartments that
empty and refill in an intact plant and effectively contribute to maintenance of whole plant water
balance remain unclear. Here, our goal was to observe the refilling dynamics of the putative
compartments for water storage (fibers, pith) and long-distance, axial transport (vessels) under
both excised and \textit{in-vivo} conditions, and to determine whether the lab-based hydraulics
methods used to study capacitance are measuring the same thing that occurs in an intact plant.
In turn, these data would then provide novel insight into the role of different tissue types in plant
hydraulic function. Experiments using non-invasive high-resolution X-ray micro-computed
tomography (microCT) imaging were complimented with traditional microscopy and
physiological measurements. Our study was performed on \textit{Laurus nobilis}, an evergreen woody
plant native to the Mediterranean region that has been documented for its ability to restore plant
hydraulic function by vessel refilling (Salleo et al., 1996, 2004; Hacke and Sperry, 2003; Trifilo et
al., 2014).
RESULTS

Excised stems

During rehydration of excised L. nobilis stems, water uptake revealed two distinct phases over a period of 20 hours (Figure 1A). The initial phase of uptake was rapid (<1 h), followed by a second phase that was relatively slow but steady. A biphasic response in water uptake was observed both in stems with and without leaves indicating that this response predominantly reflects stem internal water storage dynamics. During the initial uptake phase, $\Psi_{stem}$ recovered to values of close to zero and $\Psi_{stem}$ stabilized at >-0.05 MPa during the second phase. The biphasic response curve indicated that different tissue compartments refilled during the rehydration process (Figure 1A), and the relationship of $\Psi_{stem}$ and water uptake point to differences in hydraulic capacitance among these compartments (Figure 1B). Capacitance of tissue compartments providing for initial water storage was low (0.02 to 0.06 g MPa$^{-1}$), whereas capacitance of compartments providing for storage after >1 hour of rehydration was >10-fold higher (Figure 1B).

Tissue-specific refilling during rehydration of excised stems was visualized using microCT imaging. For a representative excised stems supplied with H$_2$O (Figure 2), fibers located in older xylem close to pith and many fibers throughout the second annual ring were initially air-filled ($A_{air-fibers}=0.09$ mm$^2$, Figure 2A, $t=2.5$ h). At the same time, many pith cells were air-filled ($A_{air-pith}=0.40$ mm$^2$), and the majority of embolized vessels were located in older xylem close to pith ($A_{air-vessels}=0.04$ mm$^2$) (Figure 2A, $t=2.5$ h). A few hours later during stem rehydration, $A_{air-fibers}$ (=0.05 mm$^2$) was reduced by 45% and $A_{air-pith}$ (=0.33 mm$^2$) was reduced by 18%; simultaneously, more embolized vessels appeared ($A_{air-vessels}=0.05$ mm$^2$) (Figure 2A, $t=7.5$ h). After 14 hours, $A_{air-fibers}$ was as low as 0.01 mm$^2$ (Figure 2A). After 18.5 hours, very few air-filled fibers remained in the most recent annual ring, while all fibers in older xylem close to pith had refilled ($A_{air-fibers}=0.003$ mm$^2$) (Figure 2A). Remaining air-filled pith cells were concentrated towards the stem center ($A_{air-pith}=0.16$ mm$^2$), and many air-filled vessels persisted in an embolized state (Figure 2A, $t=18.5$ h). These refilling dynamics were confirmed with 3-D observations during a 6.5 hour period (Figure 2B); as visualized for a portion of the stem, the air-filled volume of fibers and pith decreased from 0.006 to 0.003 mm$^3$ and 0.135 to 0.055 mm$^3$, respectively, while changes in air-filled vessel volume were negligible.

Across all excised stem samples, temporal dynamics of refilling differed among tissue compartments and among treatments when stems were either rehydrated (+H$_2$O, Figure 3A to
Figure 1: (A) Time course of water uptake (indicated by solid line) and corresponding $v_{\text{wax}}$ (indicated by symbols) during rehydration of excised L. nobilis stems. Following measurement of initial $v_{\text{wax}}$ (t=0h), the stem was connected to a water source and stem surface and leaves were sealed to prevent evaporation during rehydration. Values in legend are length and diameter of stem segments. Stems indicated in blue color (with leaves to measure $v_{\text{wax}}$) and red color (no leaves) were obtained from the same branch and analyzed simultaneously. (B) Relationship of $v_{\text{wax}}$ and corresponding amount of water uptake. For analysis of the stem indicated in red color (no leaves), $v_{\text{wax}}$ values of the stem indicated in blue color were used. Linear regression analysis allowed to determine capacitance (values in g MPa$^{-1}$) of elastic (dashed line fitted across data point 1 to 3 at most negative $v_{\text{wax}}$) and capillary (solid line fitted across data point 3 to 6) storage compartments.
areas). In excised stems during rehydration, $A_{\text{air-fibers}}$ declined rapidly (Figure 3A), while $A_{\text{air-pith}}$...
declined at a slower pace compared to fibers (Figure 3B). Simultaneously, $A_{\text{air-vessels}}$ typically
increased in the first couple of hours and decreased thereafter (Figure 3C). Curves fit to the
data using nonlinear regressions indicated that 50% of air-filled fibers refilled within around 5
hours of rehydration (Figure 3A); in comparison, a 50% reduction in air-filled pith tissue and
vessels required more than 15 and 35 hours, respectively (Figure 3B and 3C). Refilling of air-
filled fibers and pith tissue was also observed for stems that were entirely sealed (Figure 3D and
3E), but $A_{\text{air-fibers}}$ and $A_{\text{air-pith}}$ declined more slowly as compared to rehydrated stems; curves fit to
the data using nonlinear regressions indicated a 50% reduction of $A_{\text{air-fibers}}$ and $A_{\text{air-pith}}$ after
around 30h. Moreover, refilling of fibers and pith tissue was accompanied by a general increase
in embolized vessels (Figure 3F), pointing to internal water redistribution from vessels to refilling
fibers and pith in stems that were entirely sealed (for example see circle symbols); increases in
embolized vessels were least pronounced for sample ‘diamond symbols’ (Figure 3F), which
suggest that water from alternative tissue sources was also redistributed towards fiber and pith.

MicroCT images provided visual evidence that tissue-specific refilling in stems was dominated
by capillary forces, and as shown for a representative excised stem during rehydration (Figure
4): Transverse microCT images showed that the lumen of several adjacent air-filled fibers
appeared water-filled three hours later (Figure 4A). Corresponding longitudinal images indicated
that while fibers refilled, water columns expanded inside the air-filled lumen from both ends
(Figures 4B and 4C), and the liquid/air meniscus with the fiber wall was concave shaped relative
to the direction of flow (contact angle ranged from 44 ° to 63°, see also supplemental Figure S1
for fibers located close and further away from pith). For pith tissue (Figure 4D and 4E), enlarged
longitudinal images showed that air-filled cells located in proximity to xylem refilled first (Figure
4D). Refilling and water entry into the air-filled pith cell initially resulted in the formation of a
concave shaped liquid/air meniscus with the cell wall, and the appearance of a spherical air-void
before completion of refilling (Figure 4E). Refilling of the air-filled vessel lumen was related to
water droplet formations on the lateral vessel wall and water column expansion (Figure 4F); the
liquid/air meniscus with the vessel wall was variable in shape.

**Stems in intact plants**

In contrast to excised conditions, tissue-specific refilling was negligible in the stem of intact
saplings (Figures 5 and 6). In a representative *L. nobilis* sapling, $\Psi_{\text{stem}}$ recovered from -1.5 MPa
to -0.4 MPa after 20 hours of soil rehydration, but $A_{\text{air-fibers}}$ (=0.58 mm²), $A_{\text{air-vessels}}$ (=0.17 mm²)
and $A_{\text{air-pith}}$ (=0.54 mm²) remained at similar levels under *in-vivo* conditions (<6 % change)
(Figure 5A and 5B). In line with previous observations, after the stem was excised and
rehydrated for 5 hours, $A_{\text{air-fibers}}$ declined by 43% to 0.33 mm$^2$ which was accompanied by a 13%
and 23\% reduction of $A_{\text{air-fibers}}$ and $A_{\text{air-pith}}$, respectively.

For intact saplings with the shoot not bagged and exposed to ambient conditions (Figure 6A to 6C), changes in $A_{\text{air-fibers}}$, $A_{\text{air-pith}}$ and $A_{\text{air-vessels}}$ by refilling were minimal during recovery in $\Psi_{\text{stem}}$ from $-1\text{MPa}$ to $\sim -0.5 \text{ MPa}$ following soil saturation after drought. Similarly, intact saplings that were maintained well-watered and entered the experiment at less negative $\Psi_{\text{stem}}$ ($-0.5$ and $-0.4 \text{ MPa}$), no reductions in $A_{\text{air-fibers}}$, $A_{\text{air-pith}}$ and $A_{\text{air-vessels}}$ were observed; even after maintaining saplings under well-watered conditions for an additional 3-weeks (Figures 6). However, for well-watered saplings where the shoot was bagged during the time period of investigation, there was evidence for a slow but gradual reduction in $A_{\text{air-fibers}}$, $A_{\text{air-pith}}$ and $A_{\text{air-vessels}}$ over time (Figures 6D to 6F); curves fit to the data using nonlinear regressions indicated that refilling of tissues for the bagged plants resulted in a reduction of $A_{\text{air-fibers}}$, $A_{\text{air-pith}}$ and $A_{\text{air-vessels}}$ by around 20\% after 20 hours.

Stem anatomical features

Tissue viability staining showed that fibers in stem xylem were not metabolically active and dead, as well as most pith cells towards the stem center (Figure 7A to 7C). Xylem parenchyma cells were relatively inactive in their metabolic activity at the time of analysis as evident from small amounts of ray tissue emitting a green fluorescence signal. Within the pith, only cells located closest to xylem tissue (first 1 to 3 cell layers) were living (Figures 7A -C). Anatomical features of fibers, pith tissue, and vessels were further characterized using ESEM and microCT.
imaging (Figure 7D to 7F). ESEM images showed the existence of fiber-to-fiber pits (Figure 7D)

Figure 6: Temporal refilling dynamics of air-filled fibers, pith tissue and vessels in the stem of intact L. nobilis saplings. During the time period of investigation, the soil was fully saturated for all saplings. The shoot of saplings was either exposed to (A-C) ambient conditions or (D-F) covered in petroleum jelly and a humid plastic bag. Stem water potential (open symbols in panel A and D) of saplings was monitored periodically; values in legend are initial stem water potentials of saplings. As for Figure 3, dashed lines provide an estimate of tissue-specific refilling dynamics and were obtained from nonlinear regression analysis across data points of all samples (y=a*e^{b*x+c}); A, a=98, b=4E-11, c=1, R^2=1.64E-11, P=1; B, a=47, b=2156, c=2953, R^2=0.58, P=0.005; C, a=0.73, b=1002, c=201, R^2=0.63, P=0.01; D, a=72, b=8, c=24, R^2=0.63, P=0.02; E, a=60, b=1, c=5, R^2=0.80, P=0.001; F, a=12, b=421, c=195, R^2=0.66, P=0.01).
and vessel-vessel bordered pits (Figure 7E). Adjacent fibers were interconnected via pits; the lumen of fibers reconstructed in 3-D (in red color) was clearly visible through pit openings after fibers were sliced open (Figure 7F, panel 1). Images from 3-D volume renderings also indicated
that fibers were interconnected to neighboring vessels (predominantly in tangential direction). 3-
D volume renderings of the pith showed that the cell wall contained many cell-to-cell
connections (Figure 7F, panel 2).
DISCUSSION

Water storage and release has been described as an integral physiological process that contributes to whole plant water balance (e.g. Tyree and Sperry, 1989; Holbrook, 1995; Hao et al., 2013). In this study on L. nobilis, we characterized the tissue-specific refilling dynamics related to capillary water storage under both excised and intact (in-vivo) conditions, which revealed significant differences between rehydration processes that take place within an intact plant compared to those that occur in the type of stem material generally used for empirical hydraulics measurements in the lab. Time-series microCT imaging indicated that in intact saplings refilling of fiber and pith tissue was negligible over periods ranging from 20-h to 3-weeks unless the shoot of the sapling was bagged and completely covered with petroleum jelly to prevent any cuticular water loss and transpiration. In comparison, fiber and pith tissue refilled within hours in excised stems during rehydration. It can be speculated that L. nobilis, which is a Mediterranean species, would only experience these conditions infrequently during the growing season, if at all, or during rainy winter months that coincide with soil saturation, lower transpiration, and a wet canopy (LoGullo and Salleo 1988; Rhizopoulou and Mitrakos 1990). Contrary to the diurnal discharge and refilling of water from living cells in the bark (de Schepper et al., 2013), our data indicate that for small trees i) refilling of dead tissue compartments with capillary water is an exception and not the rule under in-vivo and transpiring conditions, and ii) dead fiber and pith tissue plays a negligible role in buffering the daily fluctuation in xylem tensions in an intact tree once empty (i.e. ‘single-use water reservoir’). Our conclusions are based on the dynamics of refilling from saplings studied here, but it needs to be tested if these results extend to larger trees in the field, across species that differ in stem anatomical features, and those that grow in different climate regions.

Several research groups have documented large daily cycles of capacitance discharge and subsequent recharge in trunks of large trees (e.g. Goldstein et al., 1998; James et al., 2003; Cermak et al., 2007; Hao et al., 2013). Using indirect measurement techniques to quantify water storage capacitance, these authors concluded that: i) water storage is important for maintaining short- and long-term plant water balance (e.g. Hao et al., 2013); ii) the diurnal withdrawal of water from, and refill of, internal stores is a dynamic process (e.g. Goldstein et al., 1998); iii) the exchange of water between storage compartments and the transpiration stream has a substantial influence on axial and radial stem water transport (e.g. James et al., 2003); and iv) sapwood is the most important storage site for water (e.g. Cermak et al., 2007). Our study was performed on young intact saplings, and while we cannot comment directly to the contribution of
the trunk of large trees to water storage, we have, for the first time, pinpointed the exact sites of capillary water storage while also characterizing the limitations to their refilling.

Since the process of capillary water storage in plants should follow the same biophysical rules independent of stem age, size, or organ type (for fiber refilling see discussion below), our data raise some valid concerns about the current paradigm that capillary water storage (such as in dead fibers) contributes to the daily discharge and recharge of stem capacitance. If this phenomenon extends beyond *L. nobilis* and is present in other tree species and mature trees, this would require a significant revision of our understanding of xylem structure-function. However, many open questions still remain, such as: Which capacitive tissue compartments provide a means to protect xylem function under drought? How is water re-distributed within stems to buffer daily fluctuations in xylem sap tension? Are there fiber types with specialized anatomical structures that facilitate water storage and release in certain plant species?

Our current knowledge on tissue-specific water storage is largely based on measurements of water-release and rehydration curves obtained from excised material (e.g. Tyree and Yang, 1990; Borchert and Pockman, 2005; Carrasco et al., 2014; Jupa et al. 2016). The advantage of our method is that it allows the researcher to obtain the exact volume of water released/stored for a given change in water potential, and in turn capacitance of different tissue compartments (capillary versus elastic storage) can be derived from the shape of the curve. Water-release curves provide indirect evidence for tissue-specific capacitance under excised conditions, but direct observations of tissue-specific water storage is lacking for most woody species under *in-vivo* conditions. Using microCT imaging we were not able to determine tissue-specific capacitance and volume fractions of water in different tissue regions, but we were able to visualize that substantial temporal differences in fiber refilling can exist under *in-vivo* and excised conditions, potentially complicating the interpretation of measurements on excised material. For intact *L. nobilis* saplings, our microCT data show that fibers rarely refill *in-vivo* after soil saturation suggesting that estimates of capillary water storage obtained on excised woody stems should not be considered as a significant part of the overall stem water storage capacity once these compartments have emptied in the intact plant; otherwise, this may overestimate the ‘real’ water storage capacity that the intact plant utilizes on a regular basis. Nevertheless, the exact implication of our findings for intact, mature trees in the field need to be investigated in more detail in future experiments.
Woody tree species growing in dry environments typically experience low $\Psi_{\text{stem}}$ values of $< -1.5$ MPa throughout the year, and species with a high abundance of non-living tissue and denser wood usually exhibit the most negative $\Psi_{\text{stem}}$ (Borchert and Pockman, 2005; Meinzer et al., 2009). In *L. nobilis*, $\Psi$ ranges from -0.4 to -2.5 MPa over the growing season reaching most negative values in late summer (LoGullo and Salleo, 1998; Nardini et al., 1996). Nardini et al. (2016) showed that water is lost from fibers under water stress in *L. nobilis*, but this observation was not addressed in detail and fiber refilling was not investigated. For *L. nobilis* and other tree species, this raises the question how and if compartments for capillary water storage refill? To date, microCT imaging data by Suuronen et al. (2013) provides the only visual evidence for refilling of fibers, which was dependent on environmental conditions such as high temperatures and darkness. Similarly, our data highlight that both fiber and pith refilling requires environmental conditions that presumably induce a substantial relaxation of xylem tension. Data collected here during rehydration of excised stems indicated that capillary water storage in dead fibers and pith requires a local $\Psi_{\text{stem}}$ of $>-0.1$ MPa in *L. nobilis*.

Water transport from vessels into fiber lumen and among fibers is generally considered to occur via pits (Siau, 1984). In Lauraceae species, xylem fibers are typically of the libriform-type, dead, with thick-walls, and simple bordered pits (Esau 1959; Schweingruber et al., 2011). Similarly, fibers studied here were non-septate with scanty pitting to adjacent vessels or fibers. MicroCT data obtained from excised stems that were entirely sealed (no H$_2$O supplied) showed that additional vessels embolized while fibers simultaneously refilled, pointing to water transport via pits during this process. Tyree et al. (1999) showed that vessels in *L. nobilis* are surrounded in places by paratracheal parenchyma cells that separate the vessel lumen from fibers. Because xylem cavitation in *L. nobilis* is thought to be nucleated by microbubbles entering the vessel lumen (Salleo et al. 1996), and fibers can function like transport bridges among vessels (Cai et al., 2014), it can be speculated that this layer of paratracheal parenchyma may impose an important barrier restricting the passage of air towards remaining functional vessels.

Tradeoffs between xylem efficiency and embolism safety have been linked to water storage and fiber traits in angiosperms (see review of Pratt and Jacobsen, 2016). For *L. nobilis* saplings as used here, microCT data combined with additional transpiration measurements indicated that water lost from fibers would only contribute a negligible amount of water to the transpiration stream. Calculations showed that a volume of water equivalent to the maximum volume of air-filled fibers in the stem (approximated to be on average 6\% $= A_{\text{air-fibers}}/A_{\text{stem}}$ in saplings analyzed,
see supplemental table S1), would be transpired in only 2 min during the daytime (transpiration rate of 4.0±0.4 \times 10^{-6} \text{ m}^3 \text{ h}^{-1}, measured gravimetrically from water loss of potted saplings where the soil was covered with plastic foil) and 30 min during the nighttime (0.3±0.01 \times 10^{-6} \text{ m}^3 \text{ h}^{-1}); for calculations the estimated stem volume and maximum air-filled fiber volume of saplings (leaf area was 25693±1211 mm$^2$) was 2596±73 mm$^3$ (as derived from stem height and diameter) and 159±4 mm$^3$, respectively. Since the amount of water released from fibers was relatively small compared to the volume of water transpired and fibers rarely refilled in-vivo, it can be inferred that fibers are rather ineffective in discharging water into the transpiration stream to buffer daily fluctuations in xylem tensions in *L. nobilis* saplings. However, water stored in fibers may have been large enough to be of relevance on a localized tissue level by providing water into xylem parenchyma cells, thereby allowing for turgor maintenance and cell function when xylem tensions fluctuated, but this topic needs more in-depth experimental testing.

In theory, fibers can only rehydrate if the liquid/air meniscus inside the lumen is able to overcome the negative pressure in neighboring xylem vessels ($P_x$). The negative pressure ($P$, relative to atmospheric pressure) of a water column inside a cylindrical tube can be estimated according to the Young-Laplace equation:

$$ P = 2\gamma \cos(\Theta) / r $$

where $\gamma$ is the surface tension of water (0.072 N m$^{-1}$), $\Theta$ is the liquid/wall contact angle of the meniscus, and $r$ is the radius. MicroCT images collected in this study showed that fibers had a radius ranging from 2.5 to 9 \mu m. For a concave-shaped meniscus with a contact angle of around 60°, we estimated a $P$ of -0.001 to -0.005 MPa, respectively, which suggests that only under conditions when $P_x$ is $>-0.005$ MPa will water enter the air-filled lumen via capillary forces. Given that vessels and pith cells were of larger lumen diameter than fibers, it would require a $P_x$ of $>-0.005$ MPa for those compartments to refill solely by capillarity. Such values were not obtained from indirect measurements of xylem pressure by $\Psi_{stem}$, but it can be speculated that such pressures exist locally where refilling of these tissue compartments was observed.

Pith tissue commonly suffers mechanical damage during development, and cells lose their ability to store carbohydrates and become devoid of content as stems mature (Esau 1959). Under drought and stem elongation, autolysis of pith cells is a common phenomenon leading to air-filled cell cavities (for review see Beers et al. 1997). Together, microCT and fluorescent light microscopy images provided visual evidence for the spatial distribution of dead and living pith cells in young stems of *L. nobilis*, and data indicate that most pith cells are air-filled, dead and
devoid of starch granules, while the remaining living pith cells are located at the periphery close to xylem. Our data suggest that during early stages of stem development xylem parenchyma may be the only effective site for carbohydrate storage in *L. nobilis* (Plavcova and Jansen, 2015). Furthermore, in woody stems with extensive secondary growth, the pith tissue typically becomes 'crushed' and disappears. Our microCT data on *L. nobilis* indicated that before the pith becomes 'crushed', air-filled cavities of dead pith cells can fill with water especially for excised samples. In turn, water released from dead pith cells may contribute significantly to measured water volume obtained from water-release curves; especially when dead pith tissue occupies a large fraction of the entire organ volume.

Recent literature using microCT imaging indicates that most woody plant species studied lack an efficient mechanism for short-term (days) embolism repair *in-vivo* (Brodersen and McElrone 2013; Cochard and Delzon, 2013; walnut, Knipfer et al., 2015b; redwood, Choat et al., 2015), with grapevine as the exception (Brodersen et al., 2010; Knipfer et al., 2015a, 2016; Charrier et al., 2016). However, CryoSEM data collected by Tyree et al. (1999) indicate that embolism repair in *L. nobilis* is associated with water droplets emerging from lateral walls similar to *in-vivo* observations on grapevine (Brodersen et al., 2010; Knipfer et al., 2015a, 2016). In line with these findings, the present microCT images emphasize that *L. nobilis* stems have the ability to form water droplets on lateral vessel walls and refill by water column expansion, but over the time course of investigation vessel refilling was insignificant. Hacke and Sperry (2003) reported that vessel refilling in *L. nobilis* required maintenance of plants at $\Psi_{\text{stem}} >-0.3$ MPa for 1-h by pressurizing the root system. Together with our data, it can be followed that a mechanism for short-term embolism repair by water droplet growth is mostly inactive in *L. nobilis* and water column expansion inside the vessel lumen may also be related to capillary action, similar to the refilling mechanism of dead fibers and pith cells at $\Psi_{\text{stem}}$ of close to zero.

**MATERIAL AND METHODS**

*Plant Material*

Experiments were performed on excised stem material as obtained from terminal branches of *L. nobilis* trees growing at the Arboretum, University of California Davis. Terminal branches were harvested with pruning shears about 1 m behind the shoot tip, and maintained in a sealed plastic bag containing a moist paper towel prior analysis. In addition, *in-vivo* experiments were performed on the main stem of intact *L. nobilis* saplings (ca. 30 cm in height) that were obtained
from ArtForm Nurseries (Chagrin Falls, Ohio, USA). Saplings (n=7 in total) were grown in 4-inch
diameter plastic pots filled with soil mix (equals parts of peat moss, composted bark, sand and
perlite) and maintained for 4 weeks under greenhouse conditions (approximated day/night
temperature of 8 / 25 °C, photoperiod of 15 / 9 h, relative humidity of 35 %) at University of
California, Davis. Saplings were irrigated daily with water supplemented with macro and micro-
nutrients (similar to Knipfer et al., 2015a,b). Prior to analysis, some saplings were subjected to
drought by not watering for 3-7 days while others were maintained under well-watered
conditions. Because we were not able to monitor the entire growth period of L nobilis branches
or saplings, plant material used may have experienced some level of stress resulting in air
embolism prior investigation.

**Stem water potential**

Water status of intact saplings and harvested branch material was measured with a Scholander
Pressure Chamber (Plant Moisture Stress Model 1505D, PMS Instrument Company, Albany,
OR, USA) on mature leaves that were covered and sealed with a foiled plastic bag for >30 min
(Knipfer et al., 2015a,b). The measured parameter was defined as stem water potential ($\Psi_{stem}$).

**Water uptake curves**

Terminal branches were harvested and transported to the laboratory as described in section
'Plant Material'. In the laboratory, the branch was maintained for 1-2 hours in the sealed plastic
bag to allow for $\Psi_{stem}$-equilibration. Subsequently, initial $\Psi_{stem}$ was measured on an apical and
basal leaf, which were located at opposite sides of the stem portion of interested (length of 7 to
13 cm containing six leaves). When corresponding $\Psi_{stem}$ values differed by less than 0.05 MPa,
stem surface and leaves were covered with petroleum jelly and plastic foil to prevent
evaporation, and the portion of interest was excised with a fresh razorblade from the branch.
The apical cut of the excised stems was sealed with petroleum jelly and a 2-cm piece of PVC
tubing was placed over this stem end to hold the petroleum jelly in place. The distal cut was
connected to water-filled PVC tubing that was inserted into a water-filled cylinder that was sitting
on an electronic balance (Mettler, Toledo). Stem water uptake was recorded continuously in 30
sec intervals by weight change of the cylinder. During stem rehydration, $\Psi_{stem}$ was measured on
leaves harvested from the excised stem. Water uptake was also measured for excised stems for
which all leaves were removed during sample preparation and prior analysis. Stem hydraulic
capacitance was determined from the slope (in g/MPa) of the linear portions of the relationship
of $\Psi_{stem}$ versus water uptake (see also Borchert and Pockman, 2005).
X-ray micro-tomography

Plant material was scanned at the X-ray micro-tomography facility (Beamline 8.3.2) at the Lawrence Berkley National Laboratory. Saplings were transported by car to the Advanced Light Source (ALS, Lawrence Berkeley National Lab, Berkeley, CA) less than 4 h prior to analysis. To assess the impact of initial plant water status on tissue-specific dynamics of refilling, saplings subjected to microCT analysis covered a range of initial Ψstem (-1.5 to -0.2 MPa) as measured at ALS after arrival. During the time period of microCT investigation, the soil of saplings was fully saturated with water and Ψstem was measured periodically; for some saplings, the entire shoot was coated with petroleum jelly and covered with a sealed plastic bag containing a wet paper towel to test if minimizing transpiration affects tissue-specific refilling dynamics. For visualization of stem tissue, the potted sapling was placed in an aluminum cage and the same stem portion located 2 to 3 cm above the soil was scanned repeatedly over a period of 24 h. Some saplings were transported back to the greenhouse and maintained under well-watered conditions for an additional 3 weeks before the stem portion was subjected to a re-scan.

Excised stem samples were prepared within 5 to 8 hours following branch harvest, and were either rehydrated by supplying water to cut ends or entirely sealed. For both types of experiments, excised stem samples containing no leaves were prepared as follows: Ψstem was measured on a bagged leaf of the branch that was located less than 2 cm away from the stem portion of interest. Immediately after, the branch was submerged in water, and a stem portion of 5 to 10 cm in length was cut under water with pruning shears. (i) For rehydrated excised stems, stem ends were recut using a fresh razor blade to remove air trapped at distal ends during the initial cut and in turn ensure maximal connectivity to externally supplied H2O. Following the procedure by Knipfer et al. (2016), the trimmed stem (length 3 to 8 cm) was connected to a 2 cm piece of PVC tubing on the top end and to a 2 cm piece of PVC-tubing attached to a valve (i.e. open position) on the bottom end. Tubing was sealed with the stem using high vacuum grease (976V, Dow Corning Company). PVC tubing was entirely filled with H2O, and the valve at the bottom end was closed. The stem with attached tubing was removed from the water bath and its entire surface was covered with vacuum grease to prevent surface evaporation. (ii) For excised stems that were not supplied with H2O, stems were excised under water as described above, removed from the water bath, and the entire stem surface including the cut stem ends were coated with vacuum grease and wrapped with parafilm to prevent evaporation. Following
sample preparation, excised stems were placed in a sample holder and the same stem portion about midway along the sample was scanned repeatedly over time.

Stems were scanned in a 21 keV synchrotron X-ray beam using a continuous tomography setting yielding 1025 two-dimensional longitudinal images (resolution of 3.22 μm/pixel) that were captured on a CMOS camera (PCO.edge; PCO, Kehlheim, Germany) at 350 ms exposure time. Acquired raw images were reconstructed into transverse images using a custom software plugin for Fiji image-processing software (www.fiji.sc, ImageJ) that used Octopus software (ver. 8.3; National Institutes for Nuclear Science, Ghent University, Ghent, Belgium) in the background (Knipfer et al., 2016). Longitudinal images were generated using the slice tool in the software AVIZO (ver. 6.2; Visualization Sciences Group / FEI, Hillsboro, OR).

The cross-sectional area of air-filled fibers, pith tissue, and vessels was quantified from binary images as generated from representative transverse microCT images using a semi-automated routine in Fiji software: For a time-series of microCT images, the contrast and brightness was adjusted so that air-filled tissue was clearly visible at comparable intensity ('Image-Adjust' tool). Subsequently, the xylem including pith was extracted manually using the ‘Image-Crop’ tool, and the ‘Image-Threshold’ tool was used to label exclusively air-filled tissue; a noise filter was applied ('Process –Noise –Despeckle’ tool) to remove black outlier pixels. Subsequently, labelled air-filled pith tissue was erased manually ('Paint brush’ tool), and the remaining air-filled cross-sectional area of fibers and vessels ($A_{air\text{-}xylem}$) was measured ('Analyze Particle' tool). Following this step, labelled air-filled vessels were erased manually ('Paint brush’ tool) from images and the cross-sectional area of remaining air-filled fibers ($A_{air\text{-}fibers}$) was measured ('Analyze Particle’ tool); air-filled cross-sectional area of vessels ($A_{air\text{-}vessels}$) was determined by $A_{air\text{-}xylem} - A_{air\text{-}fibers}$. By using the original binary image again, $A_{air\text{-}xylem}$ was erased and the remaining cross-sectional area of air-filled pith ($A_{air\text{-}pith}$) was measured ('Analyze Particle’ tool). The percentage changes of air-filled tissue was determined by ($A_{air\text{-}x (Scan2,3,..,n)}/A_{air\text{-}x (Scan1)}$) x 100% (subscript ‘x’ = fibers, pith tissue, or vessels). Based on these data, the rate of %-reduction in $A_{air\text{-}fibers}$, $A_{air\text{-}pith}$, and $A_{air\text{-}vessels}$ over time was estimated from the slope (% h⁻¹) following linear regression analyses. In addition, contact angles of liquid/air menisci within the lumen of air-filled tissues were measured on longitudinal microCT images using the ‘Angle tool’ in Fiji image-processing software (www.fiji.sc, ImageJ).
For 3-D visualization, stems were imaged at higher resolution (1.27 μm/pixel). The stack of microCT images was uploaded into Fiji image-processing software (www.fiji.sc, ImageJ), and a semi-automated routine was used to segment air-filled portions of each tissue type: Pith tissue was separated manually from xylem using the ‘Polygon’ tool, and both images stacks were saved separately. The ‘Image-Threshold’ tool was used to label air-filled fibers and vessels in one image stack and air-filled pith in the other image stack. For the binary image stack of air-filled fibers and vessels, the ‘size criterion’ feature as part of the ‘Analyze particle’ tool was used to separate both air-filled tissue types, and image stacks were saved separately. Image stacks, were inverted using Fiji software, uploaded into AVIZO software, and air-filled fibers, pith tissue and vessels were visualized in 3-D using the ‘volume rendering’ tool.

Stem anatomy and tissue connectivity was studied on dry stem samples scanned at very high resolution (0.96 μm/pixel). For imaging, stem samples (around 3 cm in length) were prepared from branches harvested in the Arboretum at UC Davis, and dehydrated slowly at around 30 °C for 5 days prior scanning. Dimensions of fibers, vessels, pith cells were determined from microCT images using the ‘Line’ and ‘Polygon’ tools in Fiji image-processing software (www.fiji.sc, ImageJ).

Environmental-SEM (ESEM) imaging
ESEM experiments were performed to validate anatomical observations from microCT images. Stem samples (around 5 cm in length) were excised from branches collected at the UC Davis Arboretum, placed in a plastic bag containing a wet paper towel, sent overnight to the Environmental-SEM (ESEM) facility at Yale University, stored at 4 °C, and imaged <48 h after harvest. Samples were dissected with a razor blade to expose the xylem, and fresh tissue fragments ca. 2 mm² were placed on a Peltier-cooled stage and maintained at 0.5 °C during ESEM imaging. Samples were observed at 10.0 kV with a FEI/Philips Field Emission XL-30 ESEM under true environmental mode. Water vapor was injected into the sample chamber at 4.0 Torr water vapor pressure, thereby establishing a 95 % relative humidity to prevent desiccation. With this sample preparation no sputter coating was required.

FDA-PI viability staining
Stem tissue viability was analyzed using a fluorescence-based staining assay (Krasnow et al., 2008; Knipfer et al. 2016). For analysis, two fluorescent dyes (fluorescein-diacetate ‘FDA’ and propidium iodide ‘PI’) were used simultaneously that allow a two-color discrimination between.
living and dead tissue compartments (FDA can permeate through the intact cell membrane and non-fluorescence FDA is converted into the green fluorescence metabolite fluorescein if cells are living and the esterase enzyme is active; PI cannot permeate through the intact cell membrane, and stains the cell wall or the cell nuclei if the membrane is disrupted/leaky). The FDA-PI staining solution was prepared by adding 8 µl of FDA and 50 µl of PI to 5 ml of water. For analysis, stem samples were obtained from branches harvested at Arboretum, University of California Davis. Transverse stem sections were cut free-handed using a fresh razor blade, and were immediately submerged in the staining solution for 30 min and incubated in dark at ~23 °C. Subsequently, samples were mounted on a glass slide and observed under fluorescent light (excitation filter 490 nm and 575 nm, dichromatic mirror 505 nm, barrier filter 525 nm and 625 nm) using a Leica DM4000 B LED microscope equipped with a Leica DFC7000 T 2.8 MP camera. Images were captured in <4 h following sample preparation.

Supplemental Data
Table S1. Summary of cross-sectional areas and Ψstem of samples used in microCT experiments.
Figure S1. Visualization of fiber refilling in an excised stem during rehydration for fibers that were located either close or further away from pith.

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FIGURE LEGENDS
Figure 1: (A) Time course of water uptake (indicated by solid line) and corresponding Ψstem (indicated by symbols) during rehydration of excised L. nobilis stems. Following measurement of initial Ψstem (t=0h), the stem was connected to a water source and stem surface and leaves were sealed to prevent evaporation during rehydration. Values in legend are length and diameter of
excised stems. Stems indicated in blue color (with leaves to measure $\Psi_{stem}$) and red color (no leaves) were obtained from the same branch and analyzed simultaneously. (B) Relationship of $\Psi_{stem}$ and corresponding amount of water uptake. For analysis of the stem indicated in red color (no leaves), $\Psi_{stem}$ values of the stem indicated in blue color were used. Linear regression analysis allowed to determine capacitance (values in g MPa$^{-1}$) of elastic (dashed line fitted across data point 1 to 3 at most negative $\Psi_{stem}$) and capillary (solid line fitted across data point 3 to 6) storage compartments.

**Figure 2:** Visualization of tissue-specific refilling dynamics during rehydration (at $t=0h$) of an excised *L. nobilis* stem (length ~5cm containing no leaves; indicated by circle symbols in Figures 4A to 4C). (A) Binary images were generated from transverse microCT images and show air-filled fibers (top row), pith tissue (middle row) and vessels (bottom row) in black color. Values in mm$^2$ are air-filled cross-sectional areas; lines indicate the estimated boundaries between xylem annual rings (dashed line) and xylem to pith (solid bold line). (B) Corresponding 3-D visualizations of air-filled fibers (green color), pith tissue (white color) and vessels (gold color) for a portion of the stem; values in mm$^3$ are air volumes (some of the air-filled protoxylem vessels were excluded to allow for a better view of pith).

**Figure 3:** Temporal refilling dynamics of air-filled fibers, pith tissue and vessels in excised *L. nobilis* stems that were either (A-C) supplied with H$_2$O at cut ends or (D-E) entirely sealed. Values in MPa are initial stem water potentials measured immediately prior sample preparation; all excised stems contained no leaves. Dashed lines provide an estimate of tissue-specific refilling dynamics and were obtained from nonlinear regression analysis across data points of all samples ($y=a\cdot e^{(b/(x+c))}$; A, $a=0.004$, $b=582$, $c=57$, $R^2=0.92$, $P<0.0001$; B, $a=0.005$, $b=2279$, $c=229$, $R^2=0.73$, $P<0.0001$; D, $a=46$, $b=7$, $c=8$, $R^2=0.63$, $P=0.018$; E, $a=25$, $b=56$, $c=40$, $R^2=0.60$, $P=0.02$; lines were omitted for panels C and F were a continuous trend of refilling was lacking).

**Figure 4:** Visualization of the refilling process of air-filled (A-C) fibers, (D-E) pith, and (F) vessels in an excised *L. nobilis* stem during rehydration (at $t=0h$, circle symbols in Figures 4A to 4C). Representative microCT images show water- and air-filled tissue in light and dark gray color, respectively. (A) Enlarged transverse images show adjacent air-filled fibers, and many of these fibers refilled over time (examples indicated by black arrows). (B, C) Corresponding longitudinal images show the expansion of water columns (direction of movement indicated by white arrows) inside the air-filled lumen; fibers labelled ‘a-d’ and ‘e-j’ were positioned along
dashed lines ‘B’ and ‘C’ in panel A, respectively. Liquid/air menisci with the fiber wall were concave-shaped. (D) Longitudinal images show the progression of pith refilling from periphery towards stem center (black arrows indicate examples of refilled cells). (E) Corresponding 3-D volume rendering for an enlarged portion of the pith (position indicated by white box in panel D) visualizing cell ‘a’ during refilling (dashed line indicates the cell wall). The liquid/air meniscus with the cell wall was concave-shaped. (F) Longitudinal images show refilling of an air-filled vessel. Water columns formed and expanded inside the air-filled lumen (direction of movement indicated by white arrows); black triangles indicate droplets forming on the lateral vessel wall. (f=fiber, Pi=pith, V=vessels)

**Figure 5:** (A, B) Transverse microCT images visualizing air-filled tissues in the stem of an intact *L. nobilis* sapling following soil saturation (at t=0.h) and (C) following stem excision (length ~5cm) and rehydration for 6 hours. For better orientation, examples of the same air-filled fibers and vessels are labelled in red and yellow color, respectively, and air-filled pith tissue is labelled in orange color. Values in hours (h) is time following soil saturation; values in mm² are air-filled cross-sectional areas.

**Figure 6:** Temporal refilling dynamics of air-filled fibers, pith tissue and vessels in the stem of intact *L. nobilis* saplings. During the time period of investigation, the soil was fully saturated for all saplings. The shoot of saplings was either exposed to (A-C) ambient conditions or (D-F) covered in petroleum jelly and a humid plastic bag. Stem water potential (open symbols in panel A and D) of saplings was monitored periodically; values in legend are initial stem water potentials of saplings. As for Figure 3, dashed lines provide an estimate of tissue-specific refilling dynamics and were obtained from nonlinear regression analysis across data points of all samples (y=a∙e(b/(x+c)); A, a=98, b=4E-11, c=1, R²=-1.64E-11, P=1; B, a=47, b=2156, c=2953, R²=0.58, P=0.005; C, a=0.73, b=1002, c=201, R²=0.63, P=0.01; D, a=72, b=8, c=24, R²=0.63, P=0.02; E, a=80, b=1, c=5, R²=0.80, P=0.001; F, a=12, b=421, c=195, R²=0.66, P=0.01).

**Figure 7:** Characterization of tissue viability and anatomical features in *L. nobilis* stems. (A-C) Fluorescence light microscopy images stained with FDA (green signal inside viable tissue) and PI (red signal in non-viable apoplast) solutions. Corresponding enlarged images (location indicated by white box in panel A) show that (B) fibers surrounding vessels were dead and that only xylem ray parenchyma and (C) pith cells located in close proximity to xylem were living; pith cells located further inward were dead but maintained an intact cell wall. (D-E) ESEM
images show pit connections (indicated by triangles) between fibers and between vessels. (F)

MicroCT longitudinal images show that adjacent fibers were arranged in radial rows; examples
of fiber lumen are reconstructed in 3-D and labelled in red color. Panels 1 and 2 (position
indicated by yellow box in panel F) show enlarged images of 3-D volume renderings. Fiber-to-
fiber, vessel-to-fiber and vessel-to-vessel pits in xylem (panel 1) and cell-to-cell connections in
pith (panel 2) are clearly visible; examples of pits and cell-to-cell connections are indicated by
black triangles. Examples of granules in pith cells are indicated by arrow (f=fiber, RP=ray
parenchyma, pi=pith, V=vessel).


