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Water transport, embolism recovery and water storage in trees

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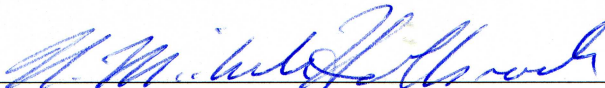
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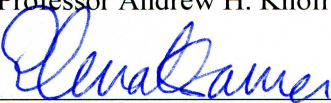
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Water transport, embolism recovery and water storage in trees

A dissertation presented

by

James Kenneth Wheeler

to

The Department of Organismic and Evolutionary Biology

In partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in the subject of

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Water transport, embolism recovery and water storage in trees

Abstract

The ability to maintain hydraulic continuity in the xylem is essential to supply leaves with the water that must be exchanged for carbon dioxide. The metastable nature of xylem sap causes this system to be inherently vulnerable to failure by rapid vaporization within the conduits. Much of the recent work on hydraulic architecture and cavitation has pursued the elusive mechanism behind apparent hydraulic recovery concurrent with tension in the bulk of the xylem, referred to as “novel refilling”. An investigation into the dynamics of this behavior (Chapter 3) revealed two key artifacts that can produce the appearance of novel refilling when in fact no embolism (and therefore, no recovery) has occurred. A further implication of these artifacts is that plant xylem may be more robust against embolism than previously expected. In the absence of novel refilling, it becomes much harder to reconcile the extreme vulnerability reported for ring porous species. Studies of *Robinia pseudoacacia* (Chapter 4) address whether the artifacts illuminated in chapter 3 provide insight into the ongoing debate about the cavitation resistance of long-vesseled species and whether it is possible to accurately assess cavitation resistance in these species using the centrifuge method. Root pressure, as an alternative to novel

refilling, provides plants with a means of reversing cavitation. Studies of *Betula papyrifera* (Chapter 5), however, show that recovery from embolism by root pressure is limited to early spring and point to an important role for water storage in fibers that minimizes xylem tensions and thus the risk of cavitation.

Overall, the studies presented here suggest a more conservative view of xylem function in which cavitation is not a regular occurrence, but solely a consequence of substantial stress, either by soil drying or freezing. This represents a departure from the current consensus that xylem embolism is highly dynamic with regular daily cycles of cavitation and refilling, and places greater emphasis on the means by which plants maintain favorable water status and the prevent cavitation.

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Chapter 1

Introduction

Hydraulic architecture, a term coined by Martin Zimmermann (1983), evaluates the anatomy and morphology of whole plant xylem through a functional lens. A central focus of functional studies of xylem transport is to understand the effects of hydraulic dysfunction through embolism and the consequences of embolism on photosynthetic function and mortality (Tyree and Sperry 1989). The dominant questions in the field revolve around the stability of the water column under normal operating conditions, how much embolism plants typically tolerate, and the mechanisms by which plants can recover from embolism. The evaluation of cavitation resistance and its importance for species' function, distribution and survival hinges on these two questions. If species can tolerate substantial embolism, or readily recover from it, embolism is of only minor importance until lethal limits are reached. If however, embolism is irreversible and any substantial embolism negatively impacts photosynthetic capacity (and competitive ability), then correctly assessing a species resistance to embolism provides insight into whole plant function and species' distributions in response to abiotic factors.

The idea that some species can recover from embolism while the bulk of the xylem remains under tension, a process dubbed “novel refilling” originated with Salleo et al. (Salleo, Lo Gullo, De Paoli and Zippo 1996; further reviewed in the background chapter). The importance of this mechanism cannot be overstated because it permits plants to stray

beyond their apparent adapted limits over short timescales and avoid catastrophic consequences. An investigation into the dynamics of this behavior in species previously reported to exhibit novel refilling (chapter 3) led to the rather startling discovery that this process does not appear to occur and that the majority of previous evidence for novel refilling may be artifactual. One of the elucidated artifacts, that cutting samples under water can introduce air into the xylem, casts doubt on measurements of native embolism in which xylem tension has not been relaxed prior to excision. By extension, it suggests that many species may experience little embolism under typical (i.e. non-drought, non-freezing) conditions. This implies that the now widely accepted concept that plants operate on the verge of hydraulic failure (Tyree and Sperry 1989) may be erroneous.

That traditional methods may artificially inflate the measured embolism in xylem sampled under tension is especially pertinent for ring-porous species. There has been considerable disagreement over the vulnerability to embolism in these long-vesseled species (Cochard et al. 2010; Sperry, Christman, Torres-Ruiz, Taneda & Smith 2012; further reviewed in the background chapter). Some previous research has implied that a substantial portion of the xylem is typically embolized, at least at midday (Taneda & Sperry 2008, Zwieniecki & Holbrook 1998), a result that researchers rationalize by the fact that ring-porous species have very high maximum wood specific conductivity and therefore may remain competitive even with a substantial portion of the xylem embolized (Hacke, Sperry, Wheeler & Castro 2006). In addition, researchers (Jacobsen & Pratt 2012) have pointed to the agreement between the degree of embolism measured in situ and the embolism predicted from centrifuge-generated vulnerability curves (in which artifacts have also been suggested to occur [Cochard et al. 2010]). The study on *Robinia*

(chapter 4) is an attempt to determine whether centrifuge curves of a ring-porous species are artificially vulnerable and if they can be incorrectly corroborated by the cutting artifact discovered in chapter 3. The data in this chapter indicate a potential for cutting artifacts even in carefully collected material, and that older material appears to be more vulnerable to embolism than current year growth when measured in the centrifuge.

In the absence of novel refilling, other methods of hydraulic recovery become more important. *Betula papyrifera* (paper birch) is known to recover from freezing-induced embolism by root pressure in the spring (Cox and Malcom 1997). What was not known was whether paper birch can recover from water stress-induced embolism throughout the growing season. A study of root pressure and trunk water content in paper birch (chapter 5) confirmed that root pressure is essential for hydraulic recovery and that root pressure ceases after leaf out, preventing paper birch from recovering from embolism during the growing season. But paper birch does not embolize naturally during the growing season, because xylem tension remains moderate. Paper birch's favorable water status is partly maintained through daily release of water from the xylem fibers, which recharge at night by capillarity. The water stored in these fibers appears to track soil water potential and diminishes slowly over the growing season.

The data from all three studies suggest a more conservative view of xylem resistance to cavitation, in which most species are adapted to minimize the embolism that occurs under typical conditions. Embolism recovery through novel refilling likely does not occur, leaving plants with only two mechanisms to restore hydraulic function. They can grow new xylem or refill embolized xylem with root pressure (Tyree & Sperry 1989). Both of these mechanisms are costly and limited to specific conditions and seasons. Therefore,

the loss of hydraulic function during the growing season can have significant fitness costs, which may not be ameliorated until the following season. In light of these costs and the data presented in these studies it seems far more appropriate to view the hydraulic pathway as adapted to minimize embolism rather than to tolerate its consequences.

Chapter 2

Background and consequences

Xylem has been described as a “vulnerable pipeline” because the fluid pressure in the conduits is below the vapor pressure of water and therefore metastable (Tyree & Sperry 1989). Water in this metastable state can vaporize rapidly, leading to emboli and rendering the conduits non-functional (Zimmermann 1983). Xylem appears to be well adapted to maintain hydraulic continuity, but some degree of embolism does occur (Tyree & Sperry 1989). It has been suggested that there may be sufficient xylem redundancy that small losses have little impact on transpirational capacity (Tyree and Sperry 1989) and that for many species, tolerating some degree of embolism may be beneficial by allowing plants to increase transpiration compared to a more conservative strategy (Sperry 2000). In addition, there is substantial evidence that recovery from embolism is possible even while the bulk of the xylem remains under tension (Meinzer, Clearwater & Goldstein 2001). Recently elucidated artifacts (Ennajeh, Simões, Khemira & Cochard 2011; Wheeler, Huggett, Tofte, Rockwell & Holbrook 2013, republished here as chapter 3) call into question whether plants really do tolerate substantial embolism under daily operating conditions, and whether recovery does occur despite tension in the bulk xylem. This background chapter outlines the impact of these artifacts and attempts to address dominant open questions related to plant water use, xylem structure and xylem resistance to embolism.

Cohesion-tension theory

Development of the consensus view of the water transport mechanism in plants has a long and contentious history chiefly because the mechanism is so counter to the behavior of water in daily life (Brown 2013). Water is drawn through a plant to its leaves by tension generated (presumably by capillarity) at the interface between the cell wall and the leaf internal air space. But while the tension required should lead to vaporization under “normal” circumstances, this typically does not occur in xylem conduits. The phase change from liquid to vapor (cavitation) can be initiated by one of two mechanisms; the separation of hydrogen bonds by fluid tension sufficient to overcome the hydrogen bond strength (homogeneous cavitation), or the interruption of hydrogen bonds by a foreign particle (heterogeneous cavitation). The theoretical tension required to initiate homogeneous cavitation at standard temperature and pressure is somewhere above 150 MPa (Zimmermann 1983), unlikely to ever occur in a living plant. Heterogeneous cavitation, however, can occur at variable tensions, which are a function of the size of the foreign particle, typically an air bubble. This is due to the tendency of surface tension at the boundary to the bubble to oppose the tension in the fluid. Smaller bubbles require greater tension to initiate cavitation. This is where plant xylem differs from our day to day experience. Xylem sap is free of bubbles because the water is ultrafiltered by the root system (passing through at least two cellular membranes at the root endodermis) and the hydrophilic walls of the xylem prevent the adhesion of air bubbles. Because the xylem conduits develop in an aqueous medium, no air bubbles exist when a conduit becomes functional. These traits maintain water in a liquid state in the xylem to tensions that are difficult to achieve in artificial systems, but the system is not perfect and under extreme

conditions portions of the xylem can be compromised by the admission of air bubbles into conduits leading to cavitation.

The tension at which cavitation occurs is species specific and tailored to the habitat in which the species resides (Pockman & Sperry 2000; Maherali, Pockman & Jackson 2004). Much of the study of xylem transport has concentrated on evaluating the site at which air is admitted (air-seeding) and the xylem anatomy that controls the pressure of failure (Cochard, Cruiziat & Tyree 1992; Sperry & Hacke 2004; Wheeler, Sperry, Hacke & Hoang 2005). The most likely source of air entry into a conduit is the inter-conduit pit membranes (Crombie, Hipkins & Milburn 1985). These sheets of modified primary cell wall separate xylem conduits and are essential to prevent a complete failure of the hydraulic system in the event of a leak. Because gas is physically incapable of sustaining tension (by definition it expands to fill the space allotted to it), any air entry into the xylem will completely fill the space it enters. To prevent total system failure the xylem is divided into hundreds of thousands to millions of xylem conduits. The loss of any single conduit is trivial as water has many pathways from the roots to the leaves (Zimmermann 1983). But this requires pit membranes to balance two conflicting requirements: preventing air entry from a cavitated conduit to a functional conduit and permitting the free passage of water between two functional conduits (Sperry & Hacke 2004). Evidence suggests that the functional trade-off does not exist at the pit level, but at the tissue level, where a greater membrane area per vessel is associated with greater hydraulic conductivity at the cost of lower cavitation resistance (Wheeler, Sperry, Hacke & Hoang 2005).

Origin of air

The fact that pits seem to be the point at which air enters the xylem raises the question: “Where does the air come from in the first place?” Some air enters the plant through damage. Branches break from wind or ice, insects puncture xylem conduits to feed. But it is likely that a majority comes from preexisting air within the plant body. All of the water in the plant body is saturated with dissolved air at atmospheric pressure. Once a gas space exists it will eventually become air-filled by diffusion (Yang & Tyree 1992). Therefore, any gas spaces created by lacunae will contain air at atmospheric pressure. The protoxylem tends to collapse as extensional growth proceeds, permitting gas entry, and this is directly connected to the functional metaxylem and then secondary xylem through the entirety of the plant body (Esau 1953). The hypothesis that protoxylem acts as the primary source of air for air seeding implies that a majority of embolism, within active xylem, should reside in the peripheral plant organs (where tensions are also greatest). However, this is difficult to evaluate in that most measurements of native embolism (the degree of embolism residing in the xylem *in situ*) are made in small branches as it is nearly impossible to remove large branches and trunk sections without inducing embolism in the process.

Maximizing photosynthetic efficiency

The loss of a conduit to embolism has traditionally been considered permanent with the exception of seasonal recovery induced by positive pressure in the xylem, either generated by moving solutes across the endodermis in the root (root pressure) (Tyree & Sperry 1989), or by a rarer, less well characterized mechanism that involves solutes in the stem xylem and necessitates freeze-thaw cycles (stem pressure). Both of these

mechanisms require the suppression of transpiration, either in leafless trees or during periods of high humidity and low light (usually shortly before dawn when ambient temperature falls below the dew point); they are generally considered relevant only to seasonal recovery from winter embolism (in trees) or more frequent recovery in herbs and vines when conditions permit. Therefore, for a large majority of plants cavitation is something best avoided. And yet several decades of research have suggested that many species operate very near the verge of critical xylem failure (Sperry 2000). Why would plants evolve to transpire with xylem tensions so near the point of no return?

Xylem tension is dependent upon a variety of factors. The availability of soil water, which is controlled by soil type, the degree of saturation, and dissolved solute concentration, will set the minimum xylem tension (Zimmermann 1983). Plants must pull harder than the soil pulls back. Also the height above the ground will contribute a minimal component. For every ten meters of height leaves must pull against 0.1 MPa, a consequence of the weight of water. But often the chief component of the tension in the xylem is due to the internal hydraulic resistance of the plant itself. Water moves through the plant because of the tension gradient established in the leaves; the greater the rate of water loss from the leaves the greater the tension gradient. Plants can minimize this gradient with greater hydraulic efficiency, but for a given individual plant with established xylem this gradient will be set by the rate of water loss (Sperry 2000). The only check on this water loss is the presence of stomata.

Stomatal guard cells control the stomatal aperture and consequently the rate of water loss from the leaf. But narrower stomatal apertures reduce the inward diffusion of carbon dioxide, and so plants must strike a balance between water loss and carbon gain (Kramer

and Boyer 1995). If water loss is too great the tension in the xylem due to hydraulic resistance will increase to the point where conduits cavitate, increasing the xylem hydraulic resistance and therefore the xylem tension. Without stomatal adjustment of water loss, this positive feedback cycle can lead to “runaway cavitation” resulting in the total loss of hydraulic function and death by desiccation (Tyree and Sperry 1988). But, if the stomata are sufficiently sensitive, some cavitation may be acceptable, as a greater tension gradient leads to greater water transport (allowing wider stomatal apertures). Therefore, operating at a tension that induces moderate cavitation may maximize carbon gain. This is the premise postulated to explain the fact that many plant species appear to operate at xylem tensions that induce embolism (Tyree and Sperry 1988, Sperry 2000). In some cases it appears that species function at tensions that induce substantial loss of their potential conducting capacity (Hacke et al. 2006), and the xylem community frequently invokes this explanation when measured xylem tension implies that substantial cavitation must occur from a species’ measured vulnerability to embolism (Zufferey, Cochard, Ameglio, Spring and Viret 2011; Johnson, Woodruff, McCulloh and Meinzer 2009). It can be a satisfying argument, but seems slightly maladaptive. Data imply that it is usually possible for a given species to have a greater resistance to embolism and still maintain sufficient hydraulic conductance. Perhaps we have systematically underestimated the resistance to embolism? The data in Wheeler et al. (2013) suggest that the classic method of measuring a species vulnerability to embolism (drying branches and measuring the relative loss of conductance due to embolism and increasing xylem tensions) can lead to an underestimation of the tension necessary to induce substantial cavitation, predicting cavitation at xylem tensions where an improved method indicates none. This artifact

appears to vary by plant species (Wheeler et al. 2013) and the extent to which it is widespread is currently unknown. In the case of *Acer rubrum*, however, it could lead to an overestimation of the degree of embolism present at midday operating xylem tension. When this observation is combined with the ongoing debate regarding the measurement of embolism by centrifugation it seems likely that many species operate with xylem tensions well below the point of any substantial cavitation (Choat et al. 2010; Cochard et al. 2010). The idea that plants would avoid substantial cavitation under normal operating conditions is more adaptively satisfying than the concept of operating on the verge of critical failure and coincides with the more traditional view that plants avoid embolism except in cases of extreme soil drying and seasonal embolism induced by freeze-thaw cycles (Zimmermann 1983).

Hydraulic recovery

It was previously stated that recovery from embolism is limited to specific conditions, namely, low or no transpiration and high soil water availability. But the last several decades have seen a flurry of research pursuing the possibility that plants can refill embolized xylem conduits even while the bulk of the xylem remains under tension, dubbed “novel refilling” (Salleo, Lo Gullo, De Paoli and Zippo 1996; Zwieniecki and Holbrook 1998; Tyree, Salleo, Nardini, Lo Gullo and Mosca 1999; Bucci, Scholz, Goldstein, Meinzer and Sternberg 2003; Hacke and Sperry 2003; Secchi and Zwieniecki 2010). This line of research was largely initiated by a report that *Laurus nobilis* plants subjected to external air pressure (mimicking the effect of xylem tension) showed recovery of hydraulic conductivity after a period of time even while the xylem remained at substantial tension (-1 to -1.5 MPa; Salleo et al 1996). This was an extremely exciting

development as it suggested that plants could indeed sustain substantial xylem embolism with only short term consequences and that hydraulic conductivity could be restored rapidly when xylem tension returned to slightly lower levels. Continued research provided further evidence that this unknown ability occurs in several species (Zwieniecki and Holbrook 1998; Bucci et al. 2003; Johnson et al. 2009; Zufferey et al. 2011). Rapid recovery of embolism induced by air pressurization was found in *Laurus* and *Populus*. Additionally, many species were shown to vary daily in the degree of native embolism present in the xylem with higher embolism at midday than in the evening or the following morning. With the mounting evidence came a plethora of potential mechanisms to explain a behavior that is difficult to envision (Holbrook and Zwieniecki 1999; Bucci et al. 2003; Hacke and Sperry 2003; Tyree et al. 1999; Nardini, Lo Gullo and Salleo 2011). The chief mechanistic issues a hypothesis needs to overcome are; whether the recovering conduits are isolated from functional xylem conduits, the mechanism by which the embolized conduits are isolated, the mechanism by which the pressure within them is increased, the metabolic source of energy to overcome the tension gradient and the mechanism for reconnecting refilled conduits without inducing further embolism (any incompletely refilled xylem conduit should instantly re-cavitate if it comes in contact with fluid under tension). While a variety of hypotheses were put forth, none could satisfactorily explain all of these points.

From an ecological standpoint the concept of daily cycles of cavitation and recovery is difficult to reconcile with the biology of plants in their environment. While novel refilling would allow plants to “overreach” their vascular system during the day and recover overnight, some part of the vasculature would be non-functional during the day (typically

the data suggest this would occur at midday). This is the very time that maximum conducting capacity is most necessary. Light levels and temperature are highest, and leaves require water both for cooling and to exchange for carbon dioxide (Kramer and Boyer 1995). The reduced hydraulic capacity created by midday embolism would exacerbate leaf water deficit and promote stomatal closure, starving leaves of carbon dioxide and raising leaf temperature. Both of these factors increase photorespiration and are detrimental to a leaf's photosynthetic apparatus (Kramer & Boyer 1995). Therefore, evening embolism recovery mechanism might restore hydraulic conductivity, but at a cost of reduced photosynthetic capacity, increased photorespiration and potentially damage done in the leaves which would require further repair. It seems unlikely that the slight increase in transpiration before the failure of the hydraulic system would contribute sufficient carbon gain to outweigh the costs of hydraulic recovery and the recovery from damage induced by light stress in the leaves.

Instead of providing a comprehensive mechanistic explanation of the behavior, the data in Wheeler et al. (2013) suggest an alternate interpretation of the data. Severing the xylem under tension, even when the excision is made under water, can induce embolism. This induced embolism increases with increasing tension. This leads to the possibility that the measured diurnal patterns of embolism are in fact measured diurnal patterns of cutting induced embolism as a function of the diurnal pattern of xylem tension. Wheeler et al. also demonstrate that cutting stems under water shortly after air pressurization leads to induced embolism. These two facts cast doubt on a large majority of the evidence for novel refilling and require a reevaluation of the species in which it has been demonstrated.

However, non-invasive imaging studies should be immune from either of the artifacts demonstrated in Wheeler et al. Several imaging studies have shown recovery from embolism in intact plants. To date these studies have been limited to circumstances in which it can be difficult to ascertain the xylem tension at the time of recovery and therefore not explicitly rule out recovery by root pressure. Magnetic resonance imaging (MRI) of a single *Vitis vinifera* plant revealed embolism reversal only after the four meter long plant had been re-watered and the lights turned off (Holbrook, Ahrens, Burns, & Zwieniecki 2001). While bleeding from a cut side branch was not observed, *V. vinifera* is known to generate root pressure. Without accurate measurements of xylem tension over the period of imaged recovery it is not possible to discount this as the mechanism of repair. More recently Brodersen et al. (Brodersen, McElrone, Choat, Matthews, & Shackel 2010) have employed high resolution CT to visualize embolism recovery (again in *V. vinifera*). Due to the spatial constraints of the imaging equipment they were limited to small (0.5 m) plants, which must be tightly wrapped during the imaging process (presumably suppressing transpiration). Re-watering seems a prerequisite for recovery, and though removed leaves indicated that the xylem tension did not become less negative than -0.5 MPa, the xylem tension at the site of recovery is still uncertain.

Brodersen et al. (2010) interpret their experiments to demonstrate refilling while much of the xylem remains under tension. However, it may not be accurate to dismiss root pressure as integral to this recovery for two reasons. First, the assumption that covered leaf water potentials are in close agreement with water potentials a few centimeters above the root collar is called into question by the fact that the potential of hydrating leaves may asymptote before reaching the source potential (Boyer 1968; Boyer 1971). Second, the

radial expansion of droplets, which the Brodersen et al. cite as a key refutation of root pressure, is not in fact inconsistent with this process. Unless the refilling conduits are open directly to the atmosphere, air bubbles will remain trapped within those conduits. Fluid of elevated pressure will be forced across all the pit membranes of the conduit causing droplets to form on the lateral walls of the conduit as opposed to a steadily rising fluid front that would occur if the conduit were open to the atmosphere. Therefore, while imaging studies are immune to the cutting artifacts elucidated in Wheeler et al. (2013), they do not provide convincing evidence of embolism recovery independent from root pressure. Further studies with carefully controlled xylem tension would help to clarify this ongoing mystery, but it seems likely that “novel refilling” has been a red herring and that further studies carefully designed to eliminate the problems discussed here will fail to demonstrate this behavior.

Xylem tension

The classic method of determining a species' vulnerability to cavitation employs long branches (longer than the maximum vessel length), which are dried in the lab until they reach a target xylem tension (Sperry, Donnelly and Tyree 1988). Measurement segments are then removed from the branch under water and the hydraulic conductance of these segments is measured. The segments are then flushed with water at high pressure to remove embolism and the conductance re-measured to determine the level of cavitation present at the tension measured in the branch (usually quantified as the percent loss of conductance [PLC] from the maximum flushed conductance). As discussed previously, embolism can be generated in these segments by the act of severing the conduits while the xylem sap is under tension, but this can be eliminated by relaxing the tension in the

branches prior to segment excision. Typically the xylem tension at the time of excision (or prior to relaxation) is measured using the pressure chamber on a leaf, but as xylem tension increases and embolism becomes prevalent leaves can become hydraulically isolated and may no longer reflect the tension in the xylem of the stem from which the measurement segment has been removed (Wheeler et al. 2013). This can be exacerbated by water released through cavitation events which can hydrate the decoupled leaves and lead to measured tensions lower than what exists in the stem xylem. This can be a pernicious problem, which can make it difficult to establish correctly the tension at which embolism occurs, especially at tensions beyond the P50. This will tend to pull a vulnerability curve toward lower tensions at high levels of embolism, implying that the xylem is more vulnerable than it actually is. In addition, plants subjected to substantial soil drying and re-watering could show tension in the leaves even while positive root pressure is refilling cavitated conduits (potentially occurring in Brodersen et al. 2010). Stem psychrometers might mitigate many of these problems, but these are not immune to decoupling and must be located near the site of study, to accurately assess xylem tension.

Vessel length

Much of the uncertainty surrounding resistance to cavitation may stem from the interaction between vessel length and vulnerability. Data from disparate methods (centrifuge curves, air injection, and single vessel air injection) all point toward the necessity of considering a species' vessel length when measuring resistance to cavitation (Cochard et al. 2010, Choat et al. 2010; Ennajeh et al. 2011). A long running debate exists regarding whether the “r” shaped curves, generated with the centrifuge technique, accurately reflect the vulnerability of long-vesseled species (Cochard et al. 2010;

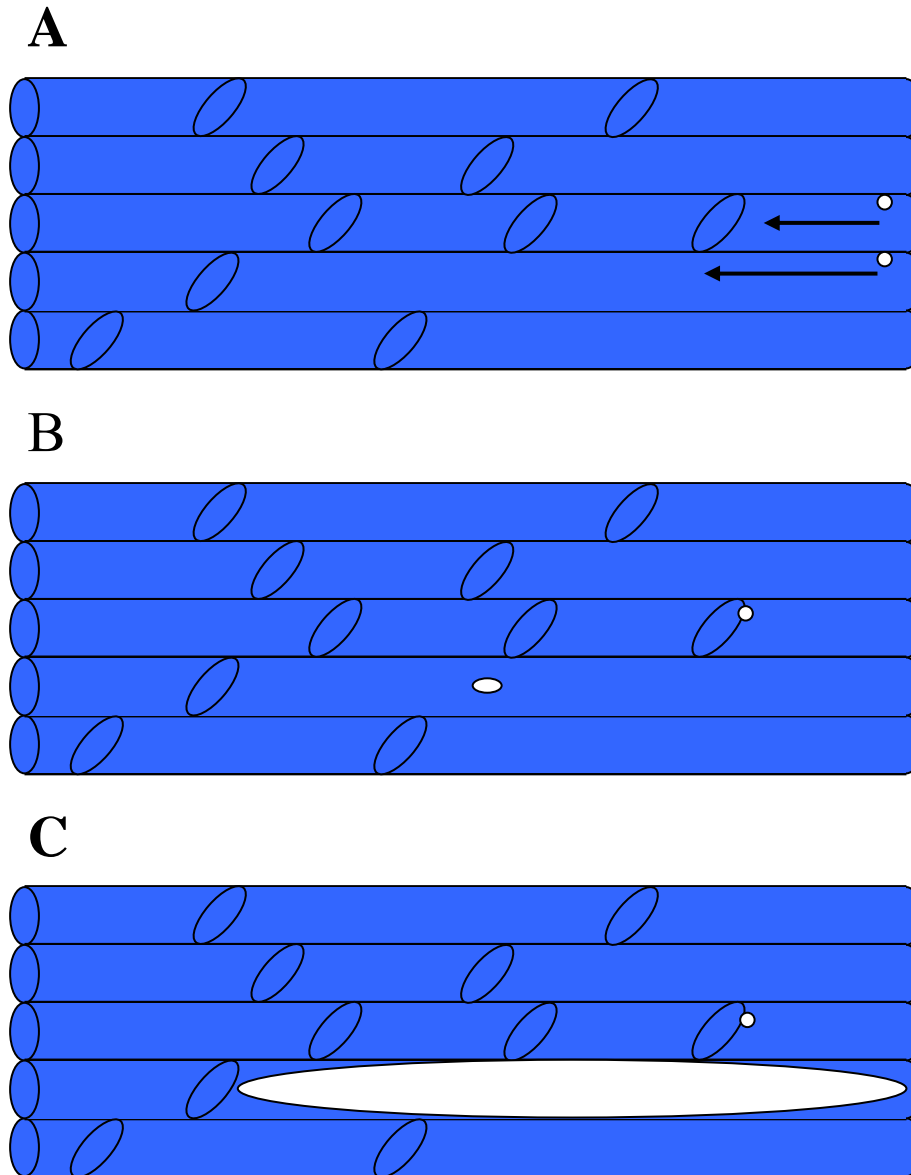
Jacobsen and Pratt 2012; McElrone et al. 2012; Sperry, Christman, Torres-Ruiz, Taneda and Smith 2012). These curves imply that for many long-vesseled species a substantial portion of the xylem is non-functional their daily operating tensions. In particular *Vitis* and *Quercus* are predicted to have greater than 60% and 80% loss of full conductivity at midday (Zuffery 2011, Tanaeda and Sperry 2008). But, native PLC measurements on these species and comparisons between different methods for measuring vulnerability to cavitation have been equivocal. Hacke et al. (2006) compared vulnerability curves generated by air injection (double ended pressure sleeve) to those generated with the centrifuge method for two species of *Quercus* and one species of *Rhus* and found close agreement between the methods. But comparisons of different methods in *Vitis* (Choat et al. 2010) demonstrated wildly different results depending upon the method, with bench dehydration yielding far more resistant curves than short stem air injections and the centrifuge method. This effect of segment length in the double ended pressure sleeve is corroborated by Ennajeh et al. (2011). Double ended pressure sleeve measurements appear to overestimate vulnerability to cavitation when the pressure sleeve is long and the stem segment short. Sperry et al. (2012) corroborate the centrifuge with native PLC measurements and a bench drying curve in *Quercus gambelii*, but it is unclear to what extent cutting artifacts may have diminished the native conductance measurements giving false confidence in the accuracy of the centrifuge method.

The potential for long vessels to complicate measurements of embolism by bench dehydration are intuitive; the longer the vessels the greater the difficulty of collecting samples sufficiently long to prevent air entry during harvest and relaxation. But the potential for long vessels to bias centrifuge and air-injection vulnerability curves is less

immediately apparent. In the centrifuge, any vessels open from the center to the cut ends may permit micro-bubbles (originating either from sample preparation or the flushing solution) to penetrate into the region of high tension at the center of the rotating segment. The pressure gradient induced by spinning should cause these bubbles to “float” toward this region of low pressure (Figure 2.1). Once, the bubbles experience sufficient tension they will initiate cavitation. Only xylem fluid filtered through inter-vessel pit membranes can safely be considered free from this potential, artificial source of cavitation. The data from Choat et al. (2010) seem to indicate the potential for this mechanism, as unflushed stems (where all the xylem fluid has been filtered through pit membranes) spun in the centrifuge indicated more resistant xylem than stems that were flushed prior to spinning. For many species (especially *Vitis* and *Quercus*) the vessels are too long to prevent a sample segment from containing a majority of vessels that are open through the segment. This may render the centrifuge technique irredeemably “broken” for measuring these species.

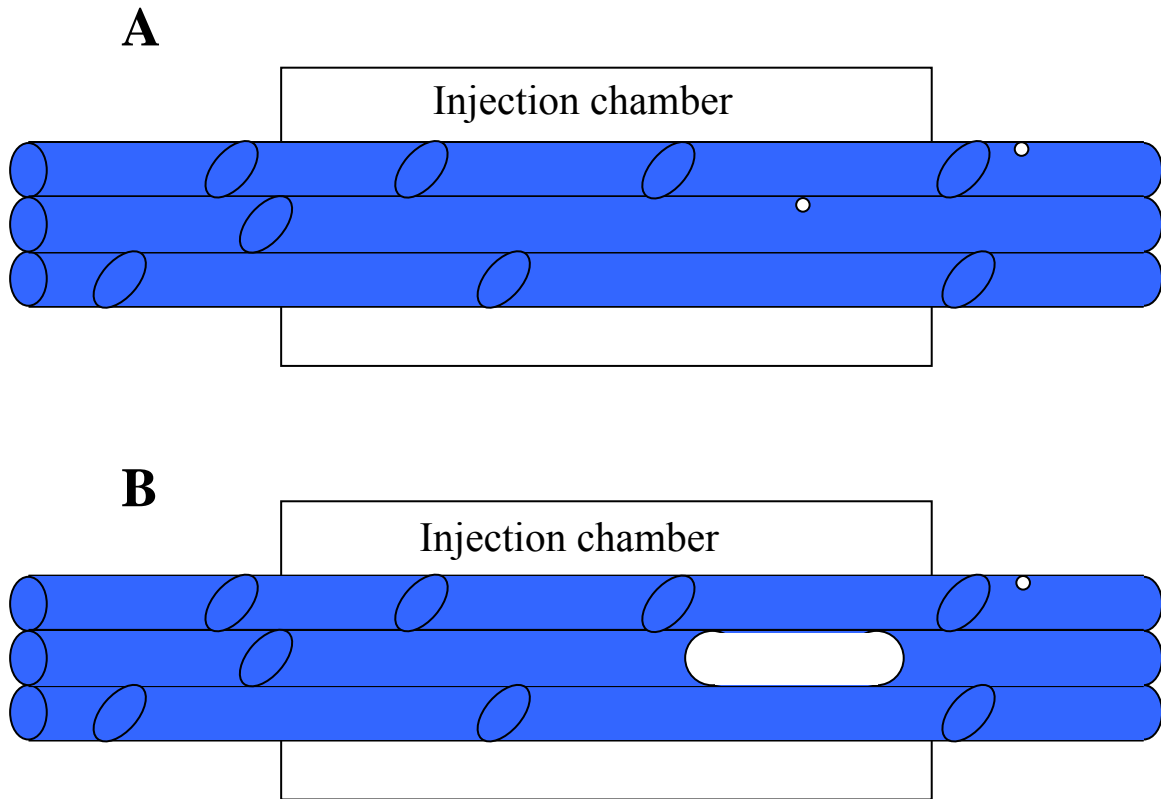
Air-injection seems prone to a similar problem when vessels are long (Ennajeh et al. 2011). When the injection chamber is near the length of the measurement segment, vulnerability curves indicate that the material is far more vulnerable than when a shorter chamber and longer segment is used. Here there is a potential for the increase in gas concentration in the xylem fluid to induce apparent embolism in any vessels that are open from the chamber to the cut end. In these vessels the concentration gradient of dissolved gas can cause any existing micro-bubbles to rapidly grow as this gas comes out of solution, which will create air filled vessels that did not originate by air-seeding (Figure 2.2).

Figure 2.1



Schematic representation of the behavior of micro-bubbles in vessels during centrifugation. A: As the tension in vessels increases, buoyancy generated by the induced pressure gradient causes bubbles to migrate toward the center where the pressure is lowest. B: In vessels with an end-wall near the cut surface the bubbles are trapped at a position where the tension is too moderate to overcome the surface tension of the bubble and it does not expand. But without an end-wall between the cut surface and the axis of rotation the bubble can travel to the center where the fluid tension is sufficient to expand the bubble (shown in C)

Figure 2.2



Schematic representation of gas exsolution generating apparent embolism during air-injection. A: prior to injection micro-bubbles introduced during flushing can exist within vessels at any point not filtered by a vessel end-wall. B: During pressurization, bubbles within the chamber can grow as the concentrated gas comes out of solution. Any bubbles that expand to fill the conduit will reduce the segment's conductivity.

Both of these potential artifacts could be verified experimentally by subjecting only those xylem conduits in which the xylem fluid has been filtered by pit membranes from the cut ends to the desired stress and then excising these portions to measure the induced PLC. This approach would, unfortunately, nullify one of the greatest benefits of both of these techniques: the ability to perform multiple measurements on a single stem. The potential for long vessels to complicate assessing xylem vulnerability to embolism and the reliability of various methods are further discussed in chapter 4.

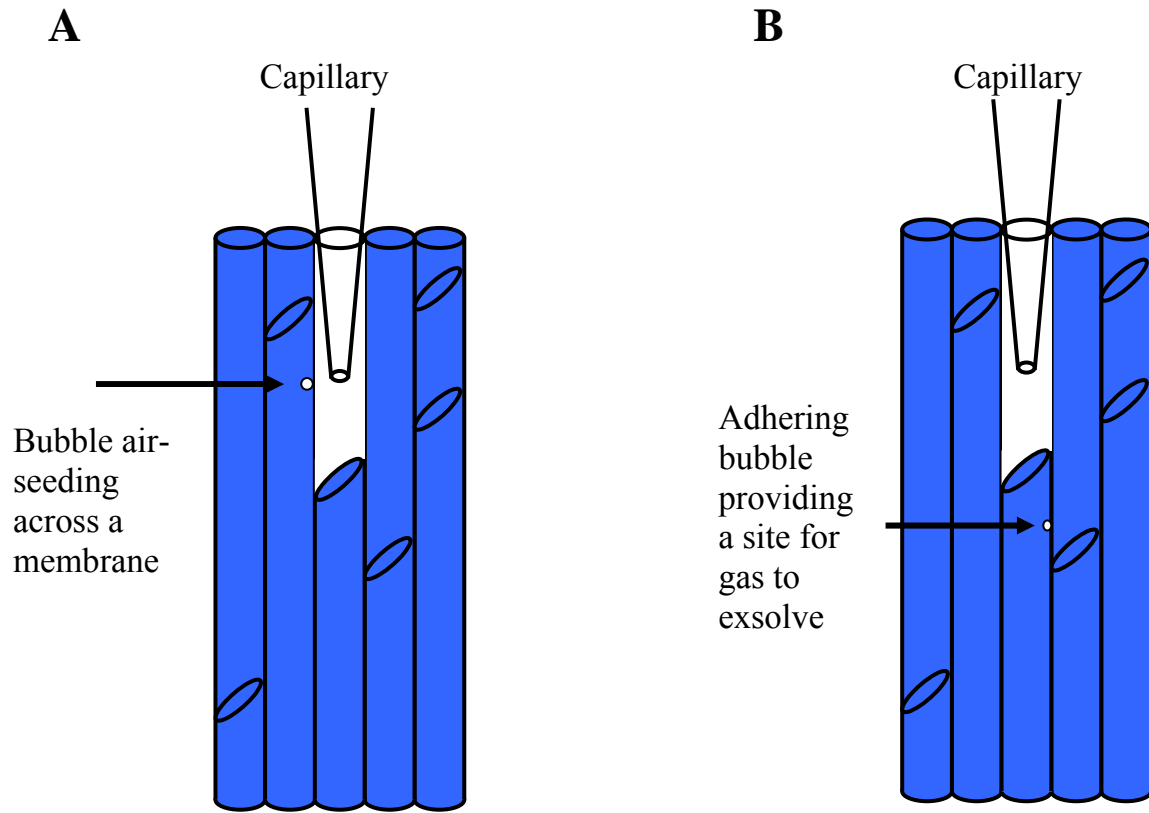
Single vessel measurements

At least one study has attempted to ascertain whole stem vulnerability by measuring the air-seed threshold of many individual vessels and then “building” a composite vulnerability curve (Christman, Sperry & Smith 2012). These measurements involve gluing a micro-capillary into an individual vessel and then pressurizing the air within until bubbles appear at the opposite end of the cut stem. A whole stem vulnerability curve can then be constructed by plotting the cumulative frequency of vessels that air-seed below a given pressure. While this approach may seem appealing it can be difficult to identify the pressure of an actual air-seeding event (Wheeler unpublished data) and the distribution of pressures measured this way appears highly sensitive to the length of the stem chosen for measurement, in relation to vessel length within the species (Jensen, Huggett & Holbrook 2013).

These two confounding mechanisms contribute to the uncertainty of scaling individual vessel measurements to the whole stem level. First, much like double-ended pressure sleeve injections, the concentration of gas in solution will rapidly increase as pressure is applied to the injected vessel. Any air bubbles in vessels open to the cut end can act as

sites for gas to exsolve, potentially leading bubble evolution at the downstream end without the occurrence of air-seeding across a membrane (Figure 2.3). This is extremely difficult to test as the bubbles may exist at any point in the stem, leading to no obvious predictable behavior. Second, it is impossible to be certain of how many membranes have been crossed for any one measurement because the length of vessels is distributed across a wide range. As the number of membranes to be crossed increases the measured air-seed pressure will be biased toward the more resistant portion of the distribution (bubbles appear when air crosses the least resistant membrane in the vessel, but if multiple vessels are in series, then bubbles will only appear when the most resistant of these vessels is breached, obscuring the lower threshold measurements). It may be possible to mitigate this bias by choosing measurement segments of an appropriate length. Jensen et al. (2013) only found convergence between the cumulative air-seed curve and a centrifuge vulnerability curve in *Acer saccharum* when the sample length for single vessel air injection was half the length used in the centrifuge. As sample length increased the distribution of measured air-seeding pressures increased, causing the stems to appear more resistant to cavitation. This bias also provides potential insight into variation in both centrifuge and air-injection generated vulnerability curves. Even without the discussed artifacts, these methods should not yield measures of vulnerability that are directly comparable between species with markedly different vessel length distributions. Shorter vesseled species will contain a higher number of membranes than longer vesseled species.

Figure 2.3



Single vessel air-injection. Bubble evolution can occur by two means; A: air-seeding can occur across an inter-vessel pit membrane resulting in an accurate measurement of the necessary pressure differential to induce cavitation or B: high pressure gas may exsolve from the fluid at any existing bubbles within a fluid filled conduit, which will cause an underestimation of the necessary pressure differential to induce cavitation.

Only bench top dehydration yields a measure of resistance to cavitation that automatically incorporates the effect of vessel length into the measurement, because the pressure gradient is applied uniformly across all the pit membranes in the branch, which is identical to the stress experienced in nature.

Water storage

The idea that plants generally prevent xylem tension from reaching a critical threshold of cavitation increases the relevance of mechanisms by which favorable water status is maintained. Obviously, stomatal closure plays a primary role in limiting water loss and preventing xylem tension from drifting into a hazardous range (Kramer & Boyer 1995). But storage within plant tissues can also mitigate xylem tensions by providing a low resistance supply of water during periods of peak demand (Shulze et al. 1985; Holbrook 1995). Zimmermann (1983) speculated on the role of capillary storage in fibers and commented that this potentially important source of water was rarely considered. In the intervening time trunk water storage has received more attention, but quantification of the volume and dynamics has been limited to estimates based upon the xylem tension and measured release curves of sample wood (Domec & Gartner 2001), or differential rates of sap flow between the crown and the bole (Goldstein et al. 1998). Tree cores may also be taken at specific time points to quantify directly the volumetric water content of the tissues. Recently, Hao et al. (Hao, Wheeler, Holbrook & Goldstein 2013, republished here as chapter 5) repurposed frequency domain reflectometry (FDR) soil moisture sensors to measure the dynamics of water content in *Betula papyrifera*. This minimally invasive technique allowed continuous observation of the dynamics of water content at a short timescale, while permitting observations to span the growing season. The data

indicate daily discharge of water from within the bole and partial recovery at night, combined with substantial recharge following large rain events. These dynamics suggest that water storage within the fibers is an integral component of plant water balance for *B. papyrifera*. The technique shows significant promise as a means of probing water storage dynamics in woody species. In addition to the insights the *B. papyrifera* study provides into the dynamics of water storage, it provides strong evidence that this species experiences limited cavitation during the growing season and that root pressure is essential for recovery from winter embolism.

Stability of xylem sap

All of the discussed artifacts and uncertainties suggest that xylem continuity may be more robust than the current consensus. While this would shift the view that xylem conductivity is highly dynamic and that species often operate on the verge of failure to a more conservative one that embolism is generally avoided, it would not diminish the importance of accurately quantifying species' resistance to embolism. Under the more conservative view that species prevent embolism, the threshold at which cavitation occurs becomes an even more important parameter as it will set the limits of a species stress tolerance. This is integral to understanding the abiotic limits of species' distributions and the way species will react to changing climate. Xylem may still be considered a "vulnerable pipeline", but perhaps not as vulnerable as it has been considered in the past.

Chapter 3

Cutting xylem under tension or supersaturated with gas can generate PLC and the appearance of rapid recovery from embolism.*

Abstract

We investigated the common assumption that severing stems and petioles under water preserves the hydraulic continuity in the xylem conduits opened by the cut when the xylem is under tension. In red maple and white ash, higher PLC in the afternoon occurred when the measurement segment was excised under water at native xylem tensions, but not when xylem tensions were relaxed prior to sample excision. Bench drying vulnerability curves in which measurement samples were excised at native versus relaxed tensions showed a dramatic effect of cutting under tension in red maple, a moderate effect in sugar maple, and no effect in paper birch. We also found that air injection of cut branches (red and sugar maple) at pressures of 0.1 and 1.0 MPa resulted in PLC greater than predicted from vulnerability curves for samples cut 2 min after de-pressurization, with PLC returning to expected levels for samples cut after 75 min. These results suggest

* reprinted from: Wheeler J. K., Huggett B. A., Tofte A. N., Rockwell F. E. & Holbrook N. M. (2013) Cutting xylem under tension or supersaturated with gas can generate PLC and the appearance of rapid recovery from embolism. *Plant, Cell & Environment* **36**, 1938-1949.

that sampling methods can generate PLC patterns indicative of repair under tension by inducing a degree of embolism that is itself a function of xylem tensions or supersaturation of dissolved gases (air injection) at the moment of sample excision. Implications for assessing vulnerability to cavitation and levels of embolism under field conditions are discussed.

INTRODUCTION

The cohesion-tension theory posits that xylem sap is under tension whenever a plant is transpiring and that the tension in the xylem is a function of the height above the ground, soil water availability, the transpiration rate of the plant, and the hydraulic resistance of the plant/soil pathway. Under periods of low soil water availability or high transpirational demand negative pressures in the xylem can “seed” the water column with tiny bubbles pulled into conduits, which can nucleate cavitation of the metastable water in the xylem conduits (Zimmermann 1983). These cavitation events disable the conduits in which they occur by blocking them first with water vapor and eventually with air (Yang & Tyree 1989). Embolized conduits diminish the hydraulic capacity of the plant, which in turn can limit its photosynthetic capability (Sperry 2000). The tension at which cavitation begins to occur appears to be well tuned to the water availability and transpirational demands of a given habitat (Choat *et al.* 2012; Lopez, Kursar, Cochard, & Tyree 2005; Pockman & Sperry 2000). Nonetheless cavitation seems to frequently occur because of drought and periods of high transpirational demand (Cruiziat, Cochard, & Améglio 2002)

Traditionally, the loss of conduits to cavitation was thought to permanently diminish the hydraulic capacity of a plant, with limited opportunities for returning embolised conduits to a functional state (Zimmermann 1983). Plants can grow new xylem and in

some cases, where transpiration is low and the soil saturated, generate a positive pressure that propagates up the xylem by transporting osmotically active solutes into conduits in the root zone. Yet, while repair by root pressure may be common in guttating herbs (Kramer & Boyer 1995), in tall woody plants its importance for stem xylem hydraulic capacity is generally believed to be limited to seasonal recovery (Ewers *et al.* 2001; Sperry, Holbrook, Zimmermann, & Tyree 1987; Sperry, Nichols, Sullivan, & Eastlack 1994). The idea that woody plants might be able to reverse embolism on a diurnal basis even when tensions in the bulk xylem are large was first put forward in the 1990's with the discovery that the percent loss of conductivity (PLC) due to embolism in air-injected samples of *Laurus nobilis* declined with time after the stems were depressurized (Salleo, Lo Gullo, De Paoli, & Zippo 1996). A new paradigm of xylem as highly dynamic, experiencing cycles of embolism and repair, has become increasingly accepted, with the ability of plants to refill under tension considered an ecologically important behavior that allows plants to operate near their hydraulic limits (Clearwater & Goldstein 2005). But to date no comprehensive hypothesis exists to explain how plants can hydraulically isolate, refill, and then reconnect conduits while the rest of the xylem remains under tension (Tyree *et al.* 1999; Holbrook & Zwieniecki 1999; Zwieniecki & Holbrook 2009).

The initial intent of this study was to evaluate the conditions under which diurnal PLC cycles occur in the hopes of better understanding the process of embolism repair. We sought to build on previous measurements (Zwieniecki & Holbrook 1998) with a larger sample size and better temporal resolution. In the 1998 study, long branches were cut from the tree in air and the segments used to measure PLC were excised promptly from the branch under water. For the current measurements, better canopy access allowed us to

make the initial cut of the branch from the tree under water, after which the branches were transported to the laboratory before excising the measurement sample. As a result, xylem tensions were largely relaxed at the time the segment for PLC measurement was cut from the branch. If cutting xylem conduits under water preserved the integrity of the water column regardless of the initial tension present, there should have been no difference in the results due to this change in protocol, and yet in red maple we did not find the pattern of diurnal PLC seen in 1998. This led us to investigate whether diurnal cycles in PLC could result from embolism formed during the excision of samples with large xylem tensions even though those cuts were made under water. We also investigated whether supersaturating water in stems that occurs during air injection could increase the likelihood of embolism formation when xylem conduits are severed.

MATERIALS AND METHODS

Plant material

Plant material included forest grown trees at Harvard Forest, Petersham, MA (hereafter, HF), as well as specimens grown on the Cambridge Campus of Harvard University, Cambridge MA (hereafter, CC). At HF, red maple (*Acer rubrum* L.), paper birch (*Betula papyrifera* Marsh.), and white ash (*Fraxinus americana* L.) were studied. The maples were mature canopy trees, approximately 30 meters tall, while the birch and ash were in a 20-meter-tall mixed stand. All HF material was harvested using a 22-m-tall canopy lift, which allowed access to the upper crowns of the trees. At CC, we employed material from a population of six red maple and five sugar maple (*Acer saccharum* Marsh) trees grown in an experimental garden, with some additional red maple material collected from three irrigated ornamental trees growing nearby. All of the trees were between five and

eight meters in height; upper canopy branches were sampled with pole pruners. We also used four-year-old sugar maple and two-year-old paper birch saplings (Lawyer's Nursery), which were potted in 5 gallon pots in Fafard 3b mixture (March 2012) and grown under supplemental lighting (14 hour days, 550 $\mu\text{mol}/\text{m}^2\text{ s PAR}$) in two greenhouses. These plants were transferred in their pots to the experimental garden in June 2012 and grown outdoors with supplemental water.

In all cases, current year extension growth from the uppermost branches exposed to full sun was sampled. We limited the measured material to current year growth to avoid embolism that originated from conditions prior to the growing season (i.e. no freeze-thaw embolism) and to facilitate the cleanest cuts possible, thus reducing the possibility that damage to the xylem during harvest would affect our results. In addition, vessels from previous years may more vulnerable to cavitation compared to current year conduits (Melcher, Zwieniecki, & Holbrook 2003).

Hydraulic parameters

Maximum vessel length was measured following the protocol of Greenidge (1952), xylem pressure was measured using a pressure chamber, and hydraulic conductance was measured onto a digital balance following Sperry *et al.* (1988). Vulnerability curves were generated for both red and sugar maple using the centrifuge method described by Alder *et al.* (1997). These methods are detailed in supplementary materials. In describing our experiments we use “relaxed tension” to refer to material supplied with water to bring xylem pressures to near zero prior to sample excision. We use “native tension” to refer to samples excised without a previous rehydration treatment. Note that in all cases, measurement segments were excised under water.

Diurnal PLC sampling

During the 2011 summer growing season, branches of HF trees were collected in the afternoon (between 1 PM and 3 PM) and again the following morning (between 5:30 AM and 6:30 AM). Two individuals of paper birch, four of red maple, and six of white ash were sampled. A split funnel was wrapped around the branch to be sampled, sealed using duct tape, and filled with water. The branch was then cut under water using razor clippers (1 megaCut S, Wolfcraft), which minimized crushing of the xylem, and the cut end immediately transferred to a bucket of water. The leafy end of the branch was covered with a large plastic bag and the branch, with its cut end remaining in water, was transferred to the laboratory. It took thirty to sixty minutes to transfer the samples to the laboratory. Based on subsequent measurements, xylem tension in the branches would have relaxed substantially before the measurements section was excised.

In maple and birch the initial cut was roughly 0.5 m from the region within the current year's extension growth that would subsequently be used for measuring PLC. When expressed in terms of vessel lengths, the distance between the initial cut and the measurement segment was at least four maximum vessel lengths for maple and at least two maximum vessel lengths for birch. In ash, the maximum branch diameter that could be severed within the water-filled funnel, coupled with much longer maximum vessel lengths (ca 1 m), resulted in collected branches in which the distance between the cut end and the measurement section was typically less than one maximum vessel length.

In 2012 diurnal measurements of PLC in red maple were repeated to directly compare the effect on PLC of cutting xylem conduits under tension. In this experiment, two branches were collected from each of four trees twice a day, in the morning (5 AM to 6

AM) and the afternoon (1 PM to 3 PM), repeated over four days. One of the branches was collected by the method described above, while the other was collected by cutting the branch in air, a minimum of three maximum vessel lengths (0.4 m) from the intended measurement segment. Immediately after the latter branch was cut from the tree, the apical end of the branch was bent into a rectangular container (20 x 30 x 15 cm) and the measurement segment excised under water, where it remained during the thirty to sixty minutes necessary to begin hydraulic measurements on both sets of samples. Note that the key distinction here is not whether the branches were cut off in air versus water, but rather the degree of tension at the time the measurement segment was excised under water.

PLC of crown-irrigated white ash

Because we were unable to harvest white ash branches that were of sufficient length to avoid the possibility of severing xylem conduits that extended into the portion of the branch used to measure PLC, we conducted an experiment at HF in which branches cut from the tree under water during the afternoon (native tension) were compared with branches cut under water following a 30 minute period during which water was supplied to the crown through a cut side branch (relaxed tension). Two adjacent branches (0.6 to 1.1 m in length) per tree from nine separate trees were harvested under water from the main axis near the apex of a tree during the afternoon. After the native tension branch was cut, the upper two meters of the crown was covered with a reflective bag to suppress transpiration and water was provided through the cut branch stub with water filled tubing that was attached to the cut end while it remained under water. After 30 minutes a second branch located within the bagged crown was cut under water (relaxed tension). Samples

used to measure xylem pressure (sealed in foil covered bags on the tree three hours prior to the experiment) were collected at the time that each branch was sampled. To ensure that the removal of these leaves did not introduce embolism into the measurement segments they were collected from a nearby branch. This experiment was conducted at the end of the 2011 summer season (late August).

Bench drying vulnerability curves

We compared bench drying vulnerability curves for red maple, sugar maple, and paper birch (CC) assembled from measurements on samples cut under relaxed versus native tensions. The red maple samples came from trees in both the experimental rain-fed garden and the irrigated population. The sugar maple and paper birch samples were taken from potted plants. Forked branches 0.6 to 1.2 meters long were cut in air and immediately enclosed in plastic bags for transport to the lab. After a thirty minute to one hour equilibration time, a short leafy shoot (a leaf for paper birch) was removed from the branch, a minimum of 30 cm (greater than one maximum vessel length for all species) from the sample segment, to determine the initial xylem pressure. To achieve a wide range of xylem tensions, some branches were air dried under lab lights ($<10 \mu\text{mol m}^{-2} \text{s}^{-1}$ PAR) for periods of up to 8 hours and then bagged for a minimum of one hour to allow xylem pressure to equilibrate across the branch.

While the branch xylem was under tension, one segment (1-to-2 internodes long) was cut underwater near the apical end of one of the forks to measure PLC (native tension). Fifteen centimeters of the base of the original branch was then removed under de-ionized water in several 2-5 cm cuts, with approximately 10-15 seconds between each cut, and the branch allowed to rehydrate for thirty minutes to two hours while the shoot end

remained sealed inside a plastic bag. Branches with more negative xylem pressures required longer relaxation periods to achieve a final pressure greater than -0.5 MPa (typically > -0.3 MPa). After relaxation, a second short leafy shoot was harvested to estimate the remaining tension in the branch with the pressure chamber, and the segment for PLC determination (relaxed tension) was cut under water near the apical end of the second fork.

PLC in petioles of red maple

We sampled petioles from trees in the experimental garden (CC) following the same forked branch protocol described above to determine whether petioles would behave similarly to branches. To determine the degree of background embolism present we sampled in the morning during rain when xylem pressures were near zero. To determine the effect of cutting under tension we sampled petioles at midday tension during clear sunny days, without supplemental dehydration in the lab (native tension) and compared these with petioles sampled only after xylem tensions were relaxed (relaxed tension). For these measurements all cuts were made under degassed water.

Rapid relaxation of stems for PLC measurement

We compared the PLC of red and sugar maple branches sampled under native tension versus sampled after rapid relaxation (< 2 min). The goal of these experiments was to avoid the possibility of some form of refilling occurring during the > 30 min rehydration intervals of the experiments described above.

Branches (> 0.5 m) containing three consecutive internodes, each > 5 cm in length, within the current year's extension growth were cut from the tree in air and dried in the lab for varying amounts of time (0.5 - 2 hrs) to reach a target xylem pressure range (-2 to

-3 MPa for red maple and -2.5 to -3.5 MPa for sugar maple). The branches were subsequently sealed in large plastic bags for thirty minutes to two hours to allow the water potential to equilibrate across the stem. After this equilibration period xylem pressure was measured on a short leafy shoot, excised from the branch a minimum of 0.4 m (more than three maximum vessel lengths) from the intended measurement segments. The entire branch was then submerged in water and the basal and apical ends of a three internode long segment were cut under water in quick succession using a pair of razor hand pruners, after which the attached leaves were sliced with a fresh razor blade between the tertiary veins to reduce the sinks for hydration and allow the xylem tensions to relax rapidly. After two minutes under water the central internode was excised (relaxed tension) and the PLC compared to that of the segment where the first cut was made (native tension). The sampling protocol is diagramed in Appendix Figure A.1.

PLC following air injection

To evaluate whether air injected samples might also be subject to embolism induced by sample excision, we examined PLC in samples excised from air injected cut branches of sugar and red maple, by excising the measurement segment either two minutes or seventy-five minutes after pressure release (detailed in supplemental methods and illustrated in Appendix Figure A.2). The latter time period was chosen, based on our observations of bubble evolution from air injected stems, as our estimate of the minimum time needed to allow the concentration of dissolved gases within the stem to return to levels near ambient. Comparisons were made at three different air injection pressures (0.1, 1, and 4 MPa). To avoid complications associated with cutting material at native xylem

tensions, all experiments were conducted on cut branches rehydrated in the lab until xylem tensions were near zero.

RESULTS

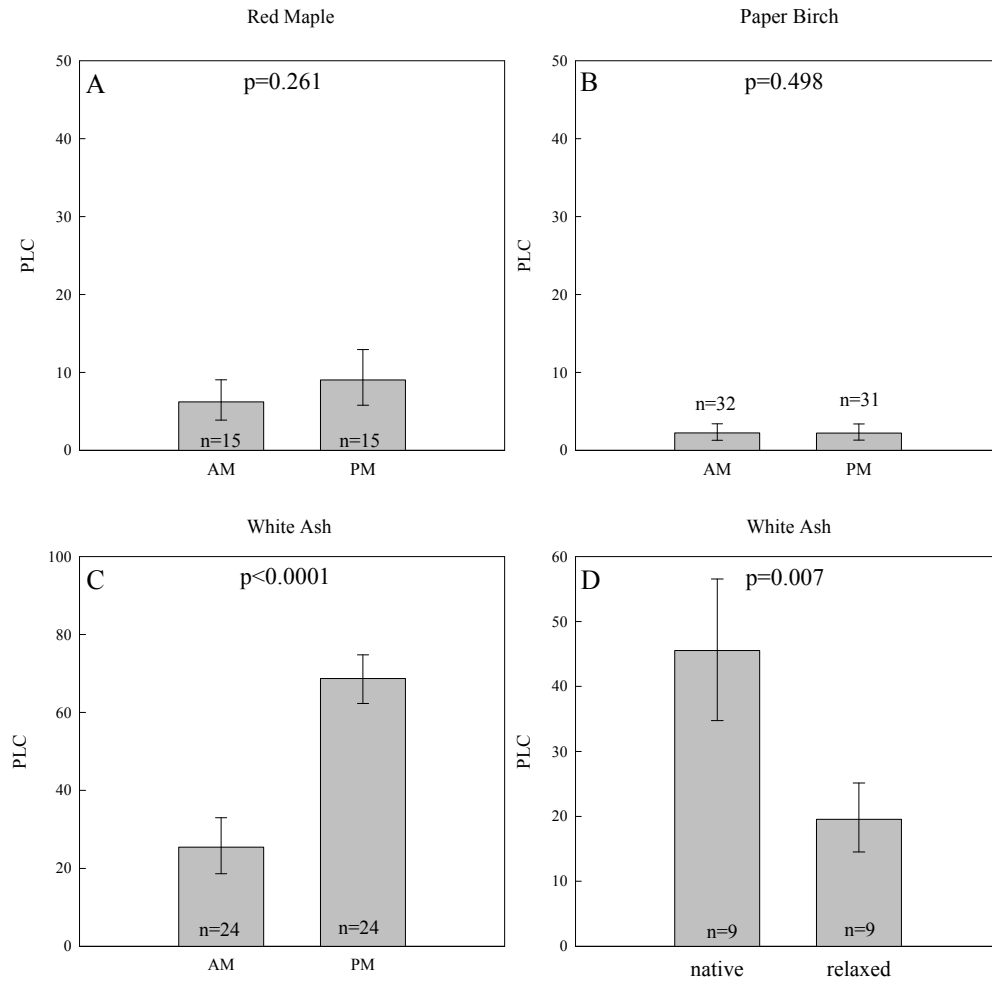
Diurnal PLC in Harvard Forest trees

The original goal of this study was to document diurnal patterns in PLC in forest trees to better understand the physiological constraints on embolism repair. We sampled three species, two of which (red maple and white ash) had been shown in a previous study to exhibit diurnal variation in PLC (Zwieniecki & Holbrook 1998). The current study differs from the previous not only in that all branches were cut from the tree under water, but more importantly that xylem tensions were allowed to relax before the segments used to measure PLC were excised.

We found that the PLC of the uppermost branches of red maple and paper birch trees sampled in this way was low and there was no significant difference between samples collected in the morning and at midday (Figure 3.1). The low midday PLC in red maple found in this study cannot be attributed to a lack of xylem tension. At midday the xylem pressures measured in red maple during the sampling days ranged from -1.8 to -2.1 MPa, similar to values reported by Zwieniecki and Holbrook (-1.1 to -2.1 MPa). Birch midday xylem pressures ranged from -1.2 to -1.7 MPa.

In 2012 we repeated the diurnal measurements of PLC in red maple trees (HF), with the difference that segments for measurement of PLC were excised at both native and relaxed xylem tensions in the morning and the afternoon.

Figure 3.1

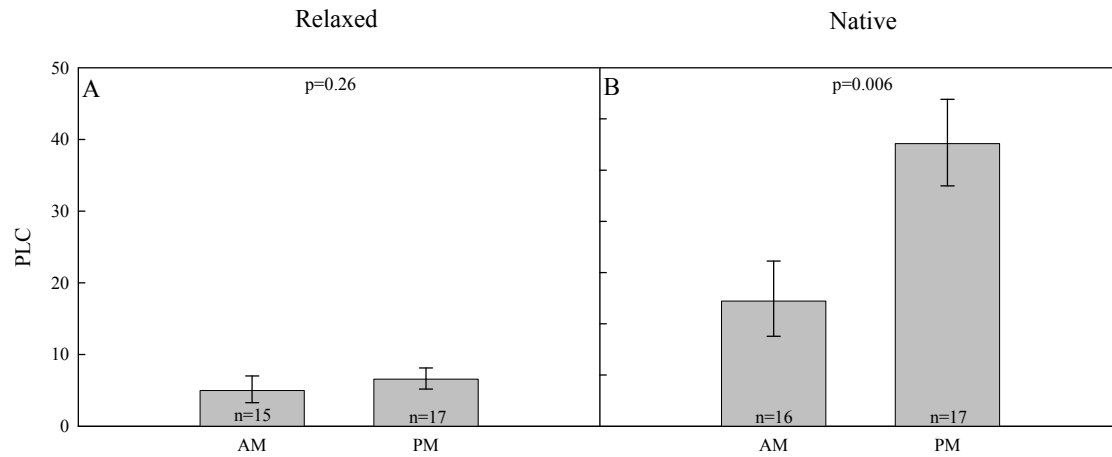


Mean (\pm standard error) PLC measured on current year growth of branches collected from the upper canopy of red maple (**A**), paper birch (**B**) and white ash (**C**) trees at Harvard Forest in the morning (5:30-6:30 AM) and afternoon (1-3PM) during summer 2011. **D**: PLC of white ash branches collected before (native) and after (irrigated) supplying water to the crown through a cut side branch for 30 minutes.

As in 2011, red maple branches cut from the tree under water and then allowed to relax fully before excision exhibited low values of PLC that were not different between morning and midday (Figure 3.2; $p=0.26$ independent samples one tailed t-test). In contrast, branches cut in air (more than three maximum vessel lengths from the region to be measured) and from which the measurement segment was immediately excised under water (i.e., at native tension) had significantly higher PLC in the afternoon than the following morning (Figure 3.2; $p=0.006$ independent samples one tailed t-test). We interpret these results as evidence that diurnal variation in PLC in red maple can be the result of embolism introduced during sample collection. We therefore believe that the measurements of PLC made on relaxed branches reflect the state in nature and thus that the red maple trees at Harvard Forest have low levels of embolism that do not fluctuate on a diurnal basis.

In contrast to red maple and paper birch, white ash had higher levels of PLC at midday (Figure 3.1C; ca. 60%, xylem pressures -2.5 to -1.4 MPa) than the following morning (ca. 30%, -1.5 to -0.3 MPa), consistent with data reported in Zwieniecki and Holbrook (1998). However, for long-vesseled taxa such as ash (maximum vessel length 1 m, versus 18 cm for birch, and 9 cm for maple), the two sampling protocols would effectively have been the same, as the relaxed samples on which PLC was measured likely contained conduits opened while still under tension during the first cut from the tree. The much shorter vessels in maple and birch meant that the initial cuts, made when xylem tensions were at their native values, occurred several maximum vessel lengths away from the measurement segment.

Figure 3.2



Mean (\pm standard error) PLC for red maple branches collected in the morning and afternoon by two different methods during summer 2012; those cut from the tree under water (**A**, relaxed) and those initially cut from the tree in air (more than three vessel lengths from the sample) and then re-cut under water (**B**, native).

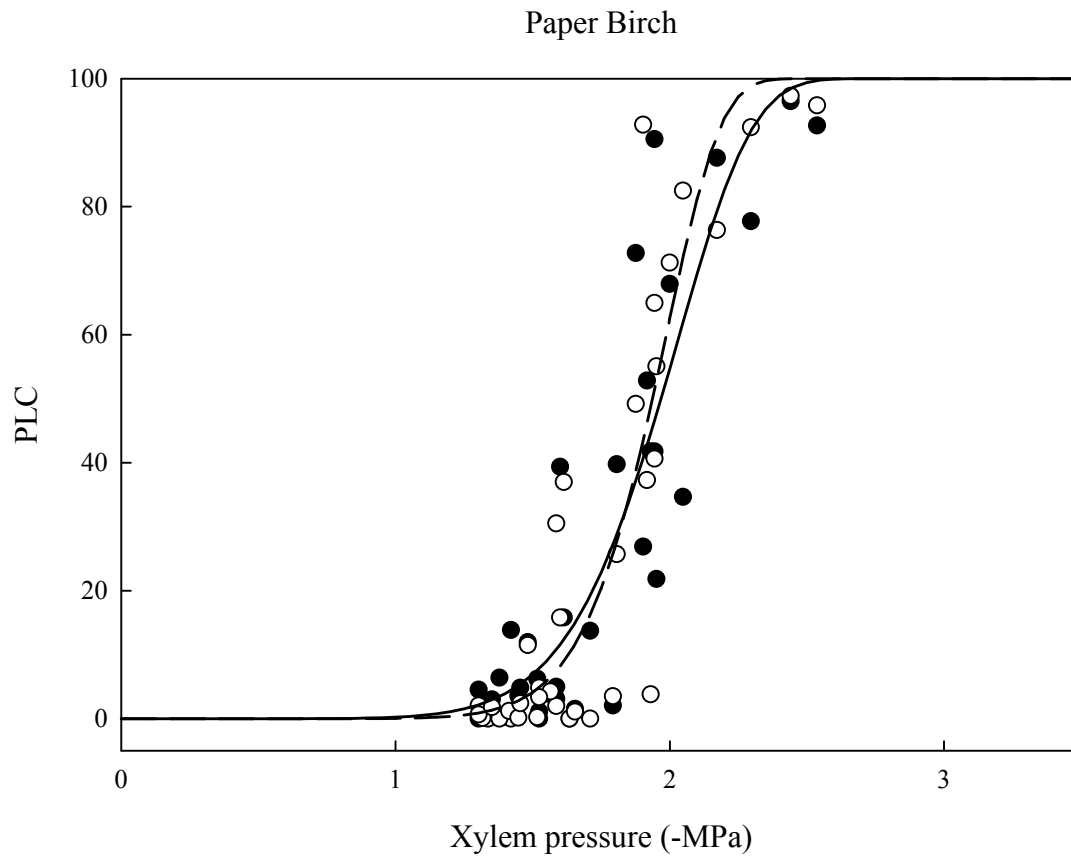
Irrigating the crowns of white ash trees resulted in a significant reduction in afternoon PLC (Figure 3.1D; $p=0.007$ one tailed, paired samples t-test). The average PLC of branches cut at native tensions was 46%, while the branches sampled following crown irrigation for 30 minutes had a PLC of 21%. Xylem pressure measured prior to irrigation was -1.0 MPa (± 0.1 MPa se), substantially wetter than measured during mid-summer. The xylem pressure following 30 minutes of crown irrigation was not significantly different from that measured prior to irrigation ($p=0.41$ one tailed, paired samples t-test). We believe this may result from the large capacitance of the crown and the distance between the water supply and the leaves used to measure xylem pressure, which was much greater than the distance between the water supply and the branch from which the relaxed sample was collected.

Vulnerability curves sampled at native and relaxed tensions

We then set out to examine systematically whether severing conduits under water affects measurements of PLC by constructing vulnerability curves using the bench drying method. In one set of samples (dried to the target xylem pressure on the bench), the measurement segments were excised under water after the imposed xylem tension had been relaxed, while in a second set of samples segments were excised at native xylem tension. These measurements were made only on the two short-vesseled species (red maple and paper birch). The long vessels in ash precluded working with individual branches and we did not have access to a sufficient number of whole trees that could be sampled to construct the two vulnerability curves.

In paper birch, we found no evidence of a sampling artifact associated with the presence of xylem tensions (Figure 3.3). Vulnerability curves in which the measurement

Figure 3.3.

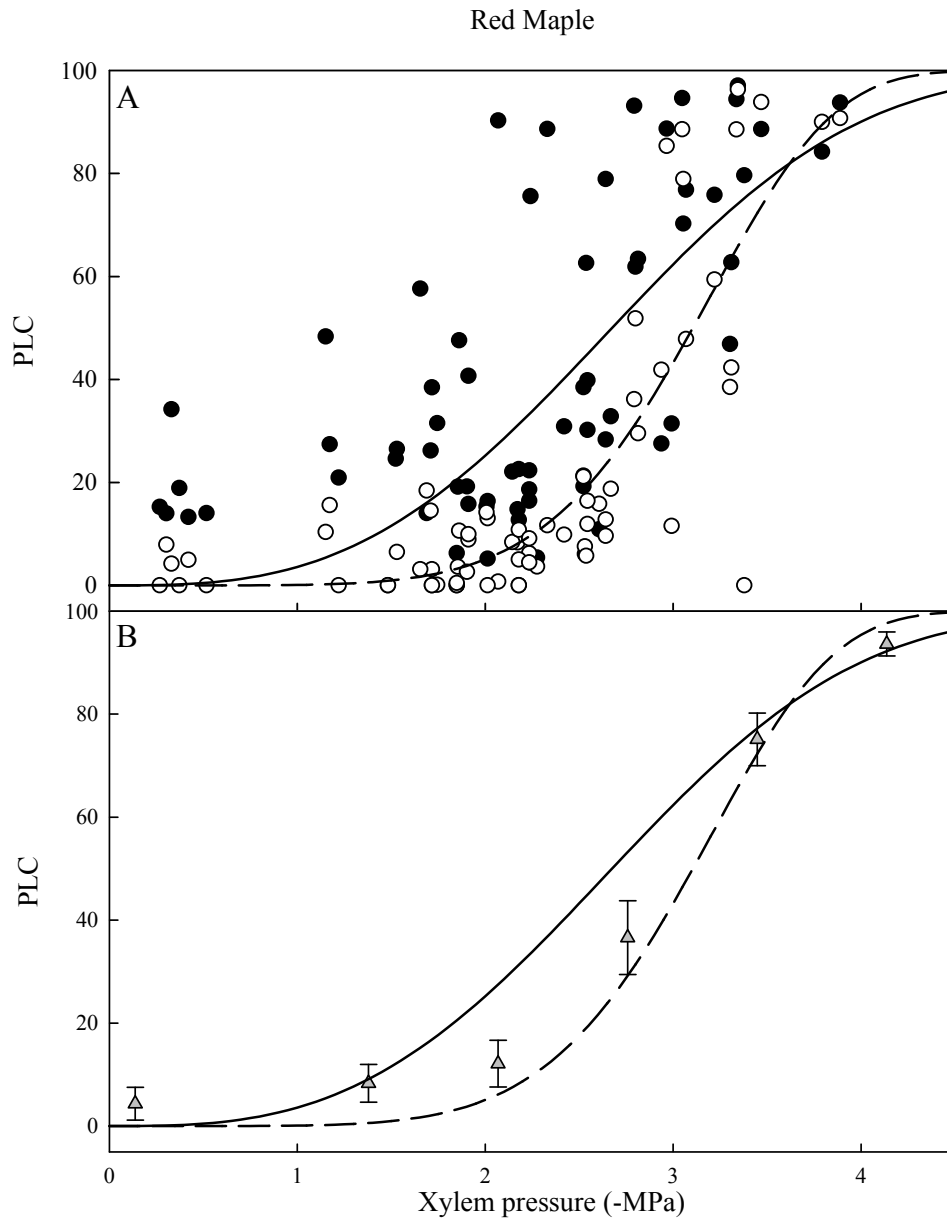


Paper birch PLC as a function of xylem pressure for segments cut under tension (native: closed symbols) and segments in which the tension was relaxed before the segment was excised (relaxed: open symbols). The data were fit by least squares regression with a Weibull function (native: solid line, $r^2=0.90$; relaxed: dashed line, $r^2=0.91$).

segments were excised at native versus relaxed xylem tensions overlapped. In contrast, red maple showed a marked discrepancy between the vulnerability curve in which the measurement segments were excised at native xylem tensions versus the curve in which tensions were relaxed prior to sample excision (Figure 3.4). In the relaxed curve, PLC remained low until xylem pressures reached approximately -2 MPa and then rose to near 100% as branches were dried to xylem pressures more negative than -3.3 MPa. In the vulnerability curve constructed from segments excised at native tensions, PLC values above background levels were observed at xylem pressures as wet as -1.2 MPa. Furthermore, between -1.2 and -2 MPa the measured PLC was highly variable, with some samples coinciding with the relaxed curve, while others exhibited PLC as high as 58%. At xylem pressures more negative than -3.3 MPa the correspondence between the native and relaxed curves was relatively good, with large (80-97%) PLC's measured in both curves.

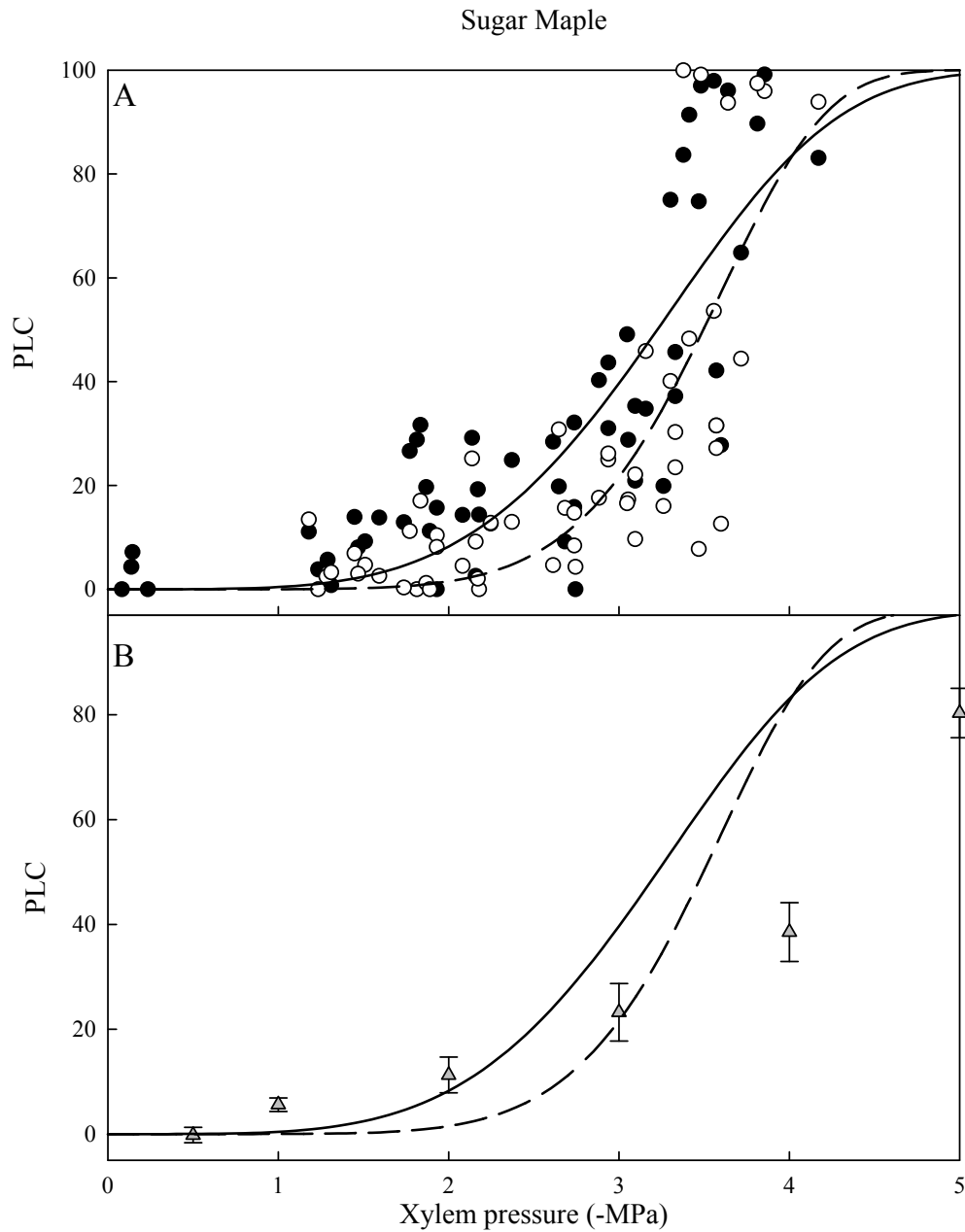
The fact that cutting under water at native tensions inflates measurements of PLC in red maple, but not in paper birch led us to question the basis for this difference. Our initial hypothesis was that paper birch cavitates at lower xylem tensions than are required to observe a discrepancy between the PLC of segments excised at native versus relaxed tensions. This motivated us to examine a second species, sugar maple, which we knew from previous work had both short vessels (< 10 cm) and was more resistant to cavitation than birch. A comparison of the relaxed versus native bench drying vulnerability curves in sugar maple shows a significant effect based on sampling method (Figure 3.5), although the overall impact is not as large as in red maple. Furthermore PLC's of sugar maple sampled at native tensions were not nearly as variable as in red maple. This

Figure 3.4



A: Red maple PLC as a function of xylem pressure for segments cut under tension (native: closed symbols) and segments in which the tension was relaxed before the segment was excised (relaxed: open symbols). The data were fit by least squares regression with a Weibull function (native: solid line, $r^2=0.66$; relaxed: dashed line, $r^2=0.72$). **B:** A vulnerability curve determined by the centrifuge method (gray triangles) is plotted as a reference (mean and standard error, $n=10$) along with the Weibull curves fit to the data.

Figure 3.5



A: Sugar maple PLC as a function of xylem pressure for segments cut under tension (native: closed symbols) and segments in which the tension was relaxed before the segment was excised (relaxed: open symbols). The data were fit by least squares regression with a Weibull function (native: solid line, $r^2=0.82$; relaxed: dashed line, $r^2=0.75$). **B:** A vulnerability curve determined by the centrifuge method (gray triangles) is plotted as a reference (mean and standard error, $n=8$) along with the Weibull curves fit to the data.

demonstrates that the potential for artifacts arising from cutting under water when the xylem is under tension is not limited to red maple. However, further work will be needed to determine what structural features of stems make one species more susceptible than another to such sampling artifacts.

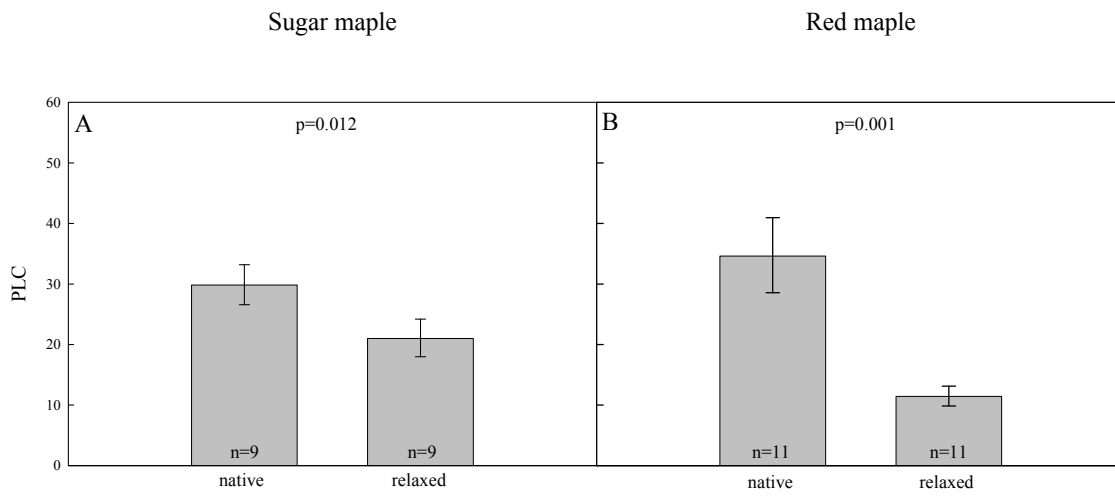
We also note that the centrifuge data and relaxed bench drying data for sugar maple diverge at xylem pressures more negative than -3 MPa. This observation combined with the slow rate at which the xylem tension of highly cavitated stems relaxed to near zero implies that the hydraulic pathway within the stem can become sufficiently limiting as to effectively decouple the xylem pressure of the leaves from that in the stems. At some point all hydraulic connections between the leaves and the stem may be severed and any water remaining in some leaves will no longer indicate the true stem xylem pressure. In addition, any stem cavitation that occurs will release water, which the leaves will take up, resulting in pressure bomb readings that indicate a less negative xylem pressure than actually exists in the stem. This suggests that in the range of xylem pressures where stems are severely cavitating, stem psychrometers may be a more reliable method of estimating xylem pressure. Specifically, we suggest that better estimates of xylem pressure at the drier end of our relaxed bench dried vulnerability curves could help resolve differences with the centrifuge curves.

At xylem pressures less negative than the P50, the point at which PLC=50, there was good agreement between the relaxed vulnerability curve generated using bench drying and the spinning vulnerability curve in both red and sugar maple. This is consistent with the idea that severing conduits while the xylem is under tension can create artifacts that inflate PLC values above *in vivo* levels of cavitation. Nevertheless, to address the

possibility that native embolism was reversed during the thirty or more minutes when the cut branches were allowed to rehydrate, we conducted a parallel set of experiments in which xylem tensions were relaxed rapidly. We dehydrated cut branches to a target range of xylem pressure (-1.91 to -2.79 MPa for red maple and -2.65 to -3.57 MPa for sugar maple) and compared the PLC in these samples to that in which tension was relaxed for two minutes (Figure 3.6). If cutting under water always preserved the hydraulic continuity of the xylem we would not expect to see a difference in the PLC of adjacent segments. Yet, in both maple species, the PLC in the relaxed segments was significantly lower compared to the segments cut under native xylem tension (Figure 3.6; one tailed, paired samples t-test: sugar maple $n=9$ $p=0.012$; red maple $n=11$ $p=0.001$). The difference between the native and relaxed values was greater in red maple than in sugar maple, as was found when longer rehydration times were used (Figures 3.4, 3.5 and 3.6).

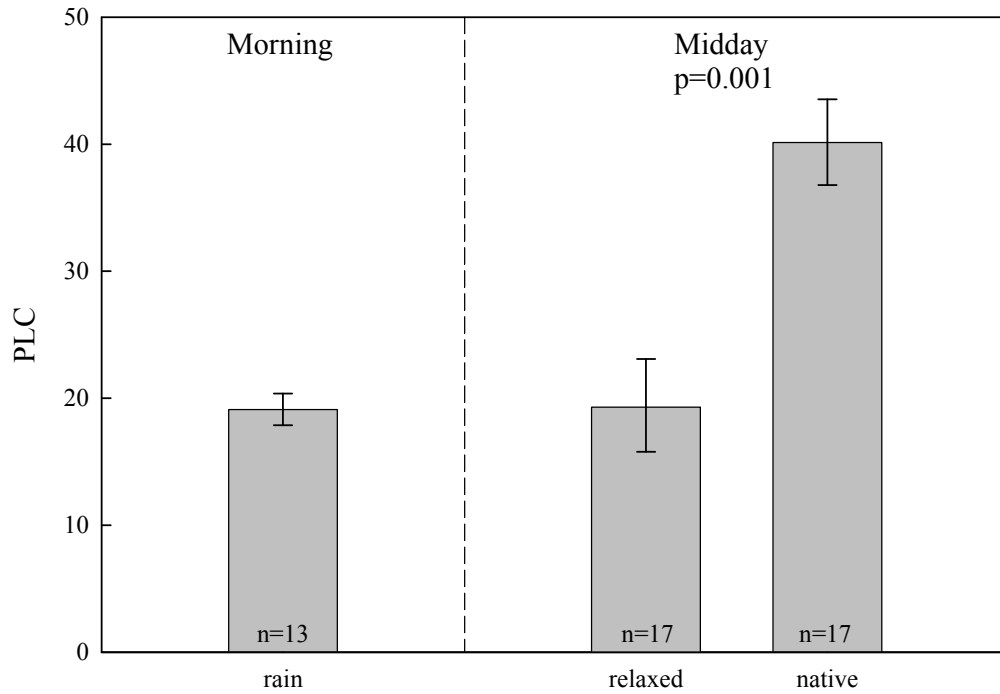
Although the focus of this study was on stems, we wanted to know whether cutting petioles under water at native xylem tensions could also lead to inflated values of PLC. To this end, we compared PLC of red maple petioles collected at midday and excised under water while the branch remained at the midday xylem pressure (-1.5 to -0.8 MPa) with petioles cut after xylem pressures had been relaxed to > -0.2 MPa. The PLC of petioles cut under water at native xylem tensions was significantly greater than the PLC of petioles where the tension was relaxed prior to sample excision (Figure 3.7; $p=0.001$ one-tailed, paired samples t-test). The PLC of petioles sampled after xylem tensions had been relaxed was not different from the background levels of PLC recorded from petioles cut early in the morning in rain ($p=0.467$ one tailed, independent samples t-test).

Figure 3.6



Mean and standard error PLC for sugar maple (**A**) and red maple (**B**) branches cut at target tension (native) and after a 2 minute rehydration (relaxed).

Figure 3.7



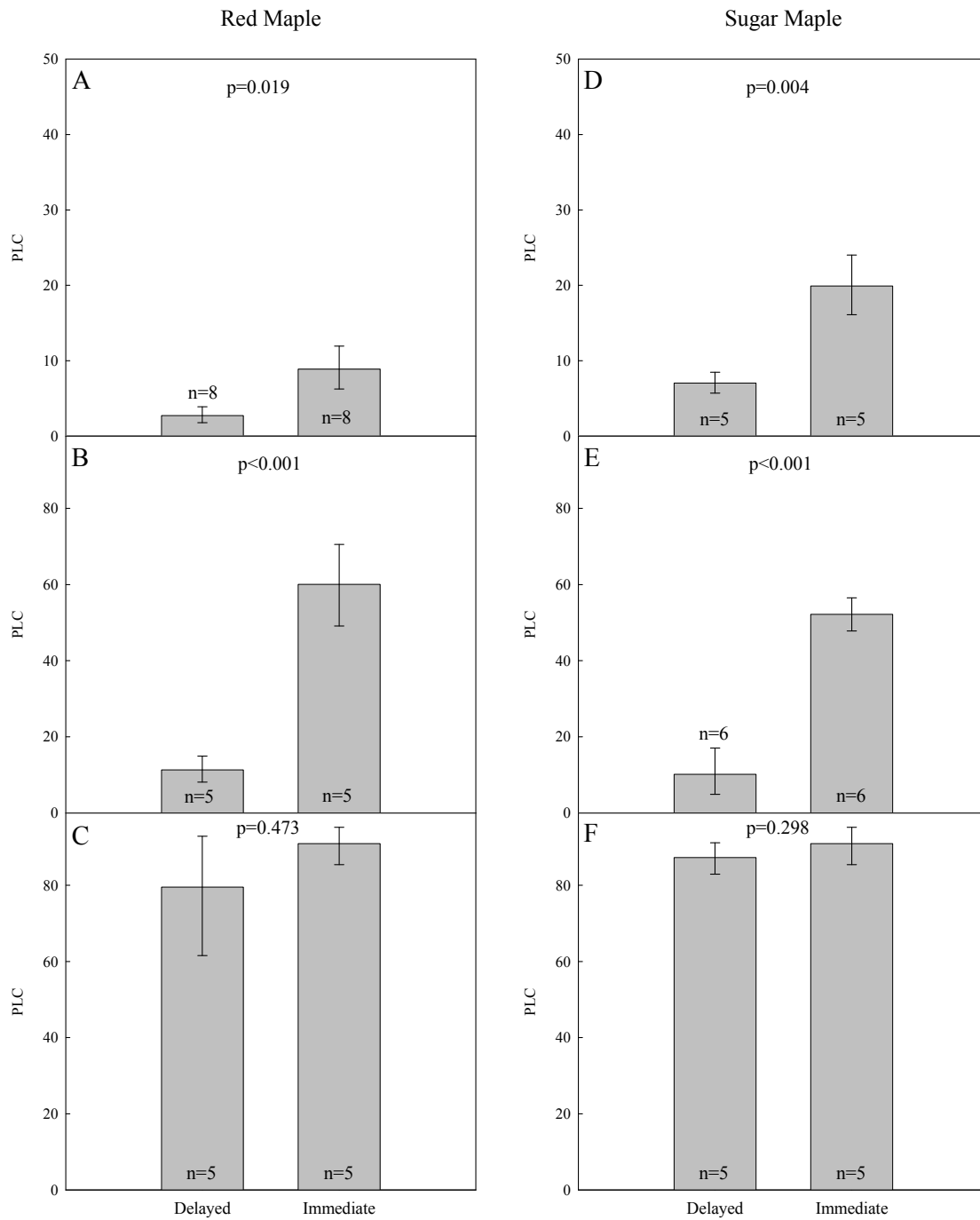
Mean and standard error PLC for red maple petioles from branches cut in the morning and at midday. Native petioles were cut under water from branches still under tension. Relaxed petioles were cut under water from branches allowed to relax to xylem pressures > -0.2 MPa. Background petioles (provided for reference) were cut under water from branches collected in the morning in the rain, xylem pressures > -0.3 MPa.

PLC following air-injection

The final part of our study evaluated whether the excision of samples following radial air injection might also inflate values of PLC due to the potential for dissolved gasses to come of out solution when a recently pressurized stem is cut. We used three different pressures, two of which we predicted, based on the centrifuge vulnerability curves, would have no (0.1 MPa) or minimal (1.0 MPa) impact on PLC. In contrast, we expected the third pressure (4.0 MPa) to cause PLC > 90 %.

Branches exposed to 1.0 MPa and then cut 2 minutes after the pressure had been released exhibited PLC's of 60% in red maple and 50% in sugar maple (Figure 3.8 B and 3.8 E), markedly higher than levels predicted from the vulnerability curves measured for each species (Figures 3.4 and 3.5; <20 % for each species). The PLC of segments excised 75 min following chamber depressurization were significantly lower than in the segments sampled at 2 min. A significant effect was present, but less pronounced, for branches exposed to 0.1 MPa (Figure 3.8 A and D). Branches pressurized to 4 MPa exhibited high PLC (Figure 3.8 C and F; 75% to 90% for red maple and > 90% for sugar maple) that was independent of the time interval between chamber depressurization and sample excision. Background PLC for all samples used in all treatments was consistently less than 10%.

Figure 3.8



Mean and standard error PLC of red maple (A, B, C) and sugar maple (D, E, F) comparing the effects of immediate or delayed sample collection following air injection at three different pressures (A, D = 0.1 MPa, B, E = 1 MPa, C, F = 4 MPa). Note that the scale for the 0.1 MPa injections has been changed.

DISCUSSION

"When a branch is cut, even under water, it is possible that bubbles are formed in the tracheae by the act of cutting. Bubbles may be formed anywhere close to the knife, but naturally mostly in the tracheae in contact with the knife on either side, as the knife introduces a discontinuity, and the water adheres feebly to it. Probably some of the bubbles observed were thus formed at the moment of making the preparation for examination, and were non-existent when the plant was transpiring." Dixon 1914, p. 94

Dixon's prescient comments notwithstanding, for the past several decades the working assumption in plant hydraulics has been that cutting under water preserves the hydraulic continuity that existed prior to cutting. Our data demonstrate that this is not always the case. In red maple, whether midday PLC was greater than that measured at first light depended entirely on how the samples were collected (Figure 3.2). When we relaxed the tensions in the xylem before excising the measurement segment, we found no diurnal variation in PLC. However, when the measurement segments were excised while there was significant tension in the xylem we found elevated PLC at midday, even though the cuts were made under water.

We interpret this as evidence that embolism can be introduced during the act of severing the vessels if the xylem tension is sufficiently large and thus that the higher midday PLC of branches sampled at native tension, even though they were cut under water, is an artifact. An alternative explanation for the differences in PLC between samples cut under relaxed and unrelaxed (i.e., native) tensions might be that refilling of cavitated xylem conduits occurred in the relaxed treatment during the 30 minute or longer

period that branches were supplied with water. We questioned this possibility for two reasons. First, the refilling under tension phenomena as currently documented appears to vanish when the phloem is damaged (Bucci, Scholz, Goldstein, Meinzer & Sternberg, 2003; Salleo *et al.* 1996; Tyree *et al.* 1999), let alone the whole stem is cut. And second, refilling by capillarity in conduits of the size found in the species examined here is too slow (Yang & Tyree 1992) to account for the differences in PLC observed here.

Nevertheless, to explore the possibility that an active (i.e., non-capillary) refilling process does indeed occur in cut branches, we undertook the rapid-relaxation experiments. In these experiments, a three-internode segment was cut at both ends under water at native tension, and after two minutes the middle internode was excised. We then compared the PLC of the middle internode (relaxed) to the segment with the end that was first cut (native), and found essentially the same difference as in the longer relaxation experiments on whole branches: higher PLC in the segment cut at native tension compared to the middle internode in which the tensions were relaxed prior to excision (Figure 3.6).

We are aware of only two material differences between the two treatments in the rapid relaxation experiment to explain the differences in observed PLC: first, the tension in the xylem at the time of the cuts; and second, the middle internode would have a greater proportion of conduits intact during the two minutes of relaxation. We considered the possibility that osmotica suspended in droplets on the walls of embolized conduits (e.g., Brodersen, McElrone, Choat, Matthews, & Shackel 2010) could provide a driving force to dissolve previously existing emboli in intact vessels, but not in opened conduits, and that this then explains the difference between the two samples. However, this hypothesis

requires (1) that the driving force for refilling not be dissipated before all of the air is forced into solution (i.e., that the reconnection problem be solved; Zwieniecki & Holbrook 2009) and (2) that the emboli be dissolved within only two minutes, substantially quicker than time scales for refilling reported in the literature (Brodersen *et al.* 2010; Hacke & Sperry 2003; Yang & Tyree 1992). Furthermore, if cut segments containing a preponderance of closed vessels are able to refill those conduits within two minutes, it would not be possible in general to measure PLC on species with short vessels. Indeed, we should not have been able to generate vulnerability curves by bench drying for either sugar or red maple. We therefore interpret the differences in PLC observed in our rapid relaxation experiments as the result of air introduced into the xylem at the moment the vessels were severed.

Our measurements of white ash are complicated by the fact that the maximum vessel length was longer than the branches available for sampling. As a result, we were unable to cut branches off under water that were more than one maximum vessel length away from the current year's extension growth. Nevertheless, when we supplied water to the stem before sampling at midday, we found significantly lower PLC, comparable to morning values (Figure 3.1D), though we were not able to document the degree of relaxation achieved in the stem xylem. These results, however, are consistent with the hypothesis that the elevated PLC that we observed at midday when sampling prior to supplying water to the stem was due to embolism introduced into the measurement segment by cutting the branch off under water.

Narrowly construed, these results simply call into question the idea that red maple and white ash undergo diurnal variation of PLC *in vivo*. However, we think such artifacts

have the potential to be widespread. When stems are cut under water, fluid accelerates into the open conduits. Micro-bubbles present in the reservoir fluid, released from the apoplast upon cutting, or arising from imperfectly wetted defects on the cutting surface (as suggested by Dixon), can be drawn into open conduits. How far a bubble penetrates into an open vessel will depend upon the rate at which the xylem tensions are relaxed, which in turn will be affected by the initial xylem tension, the conductance of the xylem, and the size of the sinks for water uptake (e.g., living cells in leaves and stems). If the tension in the water is sufficiently large, the bubbles could expand to fill the entire conduit (Appendix Figure A.3). In this case, cutting is akin to air-seeding and the open vessels would essentially cavitate.

A more likely scenario, in our opinion, is that as bubbles become entrained in the water flowing into the vessel they expand and coalesce until they become lodged against the conduit walls. In some cases bubbles may be carried as far as the vessel end wall, while in other cases, bubbles may penetrate only partway down an open conduit before becoming hung up on the vessel wall. Indeed, the absence of a cutting artifact in paper birch (Figure 3.3) may be due to their scalariform perforation plates, which may trap any entering bubbles so close to the entry-point that they were removed when the stem surface was shaved with a clean razor blade prior to measurement (John Sperry personal communication). If the scenario of artifactual PLC arising from translating bubbles is correct, then the rate at which large volume sinks for flow (such as leaves) are severed could make the difference between whether air introduced during cutting is isolated at the cut ends, where it may be trimmed off, or penetrates far into the measured sample.

Bench drying is the original method used to assess vulnerability to cavitation and it is often looked to as a reference (Christman, Sperry, & Smith 2012). Our data indicate that bench drying vulnerability curves, generated without relaxing the xylem tension in the material prior to sample excision, may indicate that plants are more vulnerable to cavitation than they truly are. In particular, we note that this artifact can lead to an underestimation of P50 and an appearance of significant levels of cavitation at moderate water potentials (Figures 3.4 and 3.5). Data from species with long vessels may be particularly at risk for bias, as the possibility of trimming out embolism caused during sample excision would be much less than in species with shorter vessels. The substantial scatter in our PLC measurements in segments cut underwater at moderate xylem tensions (Figures 3.4 and 3.5) suggests that the introduction of air during cutting may be a somewhat stochastic process affected by aspects of xylem anatomy such as conduit diameter and the prevalence of air-filled fibers or variation in cutting surfaces. Given this uncertainty, the only way to be sure that air bubbles introduced during cutting do not affect measurements of PLC is to first relax tensions and then trim off a maximum vessel length. However, Choat *et al.* (2010) were able to construct a vulnerability curve by bench drying for *Vitis vinifera* that was validated by both MRI and HRCT imaging (McElrone *et al.* 2012). In this case, once the stem had reached the target potential, leaves and lateral appendages were apparently trimmed off under water, relaxing stem xylem tensions, prior to excision of the measurement sample. We are therefore optimistic that relaxation of stems by hydration through lateral appendages (as in our ash experiments) may prove a reliable method for sampling long-vesseled species.

It has not escaped our notice that artifactually induced embolism in petioles (and likely extending into leaf veins) (Figure 3.7) may inhibit the ability to correctly evaluate leaf vulnerability to embolism as measured by either rehydration kinetics (Brodrribb & Holbrook 2003) or the evaporative flux method (Scoffoni, McKown, Rawls, & Sack 2012). However, we lack sufficient information to determine whether the resistance generated by this embolism can dominate the greater resistance of the extraxylary hydraulic pathways in the leaf. Further study will be necessary to clarify whether leaf vulnerability to cavitation can be determined accurately using existing methods.

A decrease in PLC following air-injection has been interpreted as providing a second line of evidence for refilling under tension (Salleo *et al.* 1996; Secchi & Zwieniecki 2011; Tyree *et al.* 1999). On face value, such experiments might be seen as immune from the cutting artifact described above because xylem tensions do not vary over the time period of the experiments. However, we found that injecting stems with gas, even at pressures too low to induce cavitation, inflates measurements of PLC when the vessels to be measured are severed soon after pressurization is completed (Figure 3.8). We believe that this occurs as a result of the water within stems becoming supersaturated with gases, increasing the likelihood that severing the xylem, even under very slight tensions, results in the formation of stable emboli. In other words, recently pressurized stems behave no differently than does a carbonated beverage when opened. We believe that this is why the measured PLC disappears after sufficient time is allowed for the concentration of dissolved gases to return to a level at equilibrium with the ambient air pressure. We expect that this effect is, at least to first order, independent of xylem structure and that the degree of sampling-induced embolism will be exacerbated by the presence of tension in

the xylem. As a result, we feel that all refilling data that is based on air-injection should be treated with caution.

Taken together, the artifacts documented here provide an alternative to embolism repair under tension as an explanation for changes in PLC in relation to diurnal variation in water potential or following air injection. In our minds, this calls into question the idea that embolism repair occurs concurrent with the xylem tensions found in actively transpiring plants. Whether mechanisms other than root pressure serve to reverse embolism in plants with moderate water potentials will require further work combining advanced imaging and more detailed physiological measurements on species that do not make root pressure (Brodersen *et al.* 2010).

In summary, we find that the most parsimonious explanation for our results is that severing xylem vessels that are under tension, even if the cuts are made under water, can introduce embolism into the xylem, and that the degree of embolism appears to scale with the existing tension in the xylem. Severing vessels shortly after air injection, at tensions where no embolism should occur, also appears to introduce embolism. These findings have the potential to alter our understanding of the frequency of embolism in plants in nature and the methods employed to quantify vulnerability to cavitation. If the sampling artifacts described here occur widely across species, we may need to reassess the idea that many plants operate with substantial xylem dysfunction under typical midday water potentials (e.g. Zufferey *et al.* 2011). This would call into question the idea that plants routinely operate at the brink of hydraulic failure and shift our thinking back towards the idea that, for many species, embolism may only occur to any significant degree under conditions of substantial soil drying or due to winter freezing (Zimmermann 1983).

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Chapter 4

Xylem embolism in *Robinia pseudoacacia*: drought avoidance and variable resistance to cavitation.

ABSTRACT

Xylem native embolism and xylem vulnerability to cavitation were measured in *Robinia pseudoacacia* at several periods throughout the 2013 growing season. Early season centrifuge vulnerability curves had an 'r' shape characteristic of ring-porous species, but later season vulnerability curves measured on younger material showed a sigmoid relationship. Native embolism was highly variable throughout the growing season with percent loss of conductivity (PLC) ranging from 0 to 54% with a mean of 20% and a standard deviation of 12%. Centrifuge vulnerability curves were also highly variable with the mean P50 varying from -0.4 MPa to -2.3 MPa depending on the age of the material and the time of collection. Despite this substantial variation in native PLC and vulnerability to embolism, *Robinia* appears conservative in its approach to xylem tension. During the measurement period sampled xylem tension never became more negative than -1.1 MPa and dried branches shed their leaves at approximately -2 MPa preventing measurement at more negative xylem pressures. Mean and maximum vessel length were also highly variable with the mean vessel length appearing to be a function of shoot architecture (leader shoots had longer vessels than side shoots). Long vessels have the potential to skew vulnerability data, both by cutting artifacts for bench drying and an

open vessel artifact in the centrifuge. The data here imply that even relaxed bench drying curves may exhibit some artifactual embolism and that the centrifuge may coincidentally corroborate these data through a separate artifact resulting from embolism induced in open vessels.

INTRODUCTION

Xylem resistance to embolism is considered a key feature constraining plant distributions, resistance to drought and, more generally, the conditions under which photosynthesis is possible (Zimmermann 1983, Sperry 2000). Xylem embolism due to water stress occurs when the tension in the xylem sap becomes sufficient to draw air into the xylem conduits, presumably across the inter-vessel pit membranes, and “air-seeds” a rapid phase change from liquid to vapor (Zimmermann 1983). Several methods exist to induce embolism by altering the pressure gradient between the xylem fluid and the atmosphere, allowing researchers to quantify the impact of increasing xylem tensions on a plant’s ability to conduct water in the xylem.

The original method of “bench-top dehydration” consists of cutting branches from a plant that are longer than the maximum vessel length and drying them until they reach the desired xylem tension (Sperry, Donnelly & Tyree 1988). At this point the branches are excised under water and the hydraulic conductivity is measured (native conductivity, K_n). The stems are then flushed with water (or placed under water in vacuum) and the conductivity re-measured (maximum conductivity, K_{max}). The degree of embolism is then usually quantified as the percent loss of conductivity ($PLC = 1 - K_n/K_{max}$). This method should mimic the conditions the xylem experiences in situ, that is, a uniform tension in the xylem and atmospheric pressure of the gas that is drawn into the conduits (and the gas

concentration dissolved in the xylem sap). Two slightly more artificial methods exist to induce embolism under conditions imposed by a researcher, both of which allow for repeated measurements on the same sample segment at increasing degrees of stress. Air-injection takes advantage of the fact that only the pressure gradient across the pit membranes is important in determining whether air-seeding will occur; therefore it is possible to induce air-seeding by increasing the gas pressure around the stem instead of reducing the pressure in the xylem (Cochard, Cruiziat & Tyree 1992, Sperry & Saliendra 1994). This differs from bench-top dehydration in that the gradient is imposed only locally at the site of pressurization, and that the concentration of dissolved gas in the fluid will increase above the concentration at atmospheric pressure. The third technique employs the fact that centrifugal force in a column of water rotating perpendicular to the axis of the column will induce tension at the center as a function of the density of water, the length of the water column and the speed of the rotation (Pockman, Sperry & O'Leary 1995). A stem can be placed in a custom made centrifuge rotor and spun to impose the desired tension. But the tension is not constant across the stem and is instead parabolic, with the minimum pressure occurring at the axis of rotation, while the cut ends immersed in water are at equilibrium with the atmosphere.

Generally, for a given species, these three methods indicate comparable vulnerability to embolism, but for several species, especially those with long vessels, inconsistencies arise. Often centrifuge and air-injection generated vulnerability curves of these species exhibit characteristic 'r' shaped curves indicating substantial embolism at moderate xylem tensions, which are experienced on a daily basis (Hacke, Sperry, Wheeler & Castro 2006; Li, Shi, & Shao 2010; Zufferey, Cochard, Ameglio, Spring, & Viret 2011).

Bench drying is infrequently used for these species, because the long vessels require the sampling of exceptionally long branches. Native embolism data sometimes confirm (Taneda & Sperry 2008; Tobin, Pratt, Jacobsen & De Guzman 2012, Zufferey et al. 2011) and sometimes conflict (Choat, Drayton, Brodersen, Matthews, Shackel, Wada & McElrone 2010; Cochard, Herbette, Barigah, Badel, Ennaleh & Vilagrosa 2010) with the degree of embolism predicted by centrifuge or air-injection vulnerability curves at the tensions recorded at the time of harvest.

The debate regarding the validity of these 'r' shaped vulnerability curves has generated a substantial body of literature on both sides (Choat et al. 2010, Christman, Sperry & Smith 2012; Cochard et al. 2010; McElrone et al. 2012; Sperry, Christman, Torres-Ruiz, Taned & Smith 2012; Tobin et al. 2012). Several authors cite the tendency for measured native embolism and air-injected vulnerability curves to loosely agree with these centrifuge generated vulnerability curves bolstering the idea that these 'r' shaped curves are biologically accurate (Hacke et al. 2006; Tobin et al. 2012, Zufferey et al. 2011). However, this coincidence of data from separate techniques may no longer ensure their veracity. Data presented in Wheeler et al. (Wheeler, Huggett, Tofte, Rockwell & Holbrook 2013) demonstrate that the act of cutting xylem conduits while the xylem sap is under tension can induce PLC, and data presented by Ennajeh et al. (Ennajeh, Simões, Khemira & Cochard 2010) demonstrate that air-injected stems may have artificially high levels of embolism if the injection chamber is long relative to the length of stem that is measured. These two artifacts may coincidentally produce similar, incorrect, vulnerability curves for a given species.

We sought to identify whether a cutting artifact might lead to apparent ‘r’ shaped native embolism data in the long-vesseled species *Robinia pseudoacacia*, and whether we could generate ‘r’ shaped vulnerability curves by the centrifuge technique and then possibly eliminate them through methodological modifications. We chose this species based upon its reported ‘r’ shaped curves (Li et al. 2010) and its tendency to produce long growth every season, which was imperative to allow the evaluation of material not subject to potential freezing induced embolism.

MATERIALS AND METHODS

Plant material and collection protocol

All material was collected from a rain fed population of mature (15 meter) *Robinia pseudoacacia* trees on the Harvard campus (Cambridge, MA). A majority of the samples were collected from upper canopy branches using a self-propelled boom lift (JLG 450A, JLG Industries, Inc. McConnellsburg, PA) in early August, with some supplemental branches collected with pole pruners both before and after this period. The material was collected from June through October, 2013. Except where indicated, current year branches were used. There was a pronounced heat wave in mid-July during which no rain fell and the daily high temperature exceeded 30° C.

Collection protocol

Two types of collection protocols were employed. Wet samples were wrapped in large plastic bags before excision; a funnel was taped to the branch and filled with water. The branch was then cut under water and the cut base immediately placed in a bucket of water for transport to the lab. Dry samples were cut from the tree without the funnel and immediately placed in a large plastic bag for transport to the lab. In both cases the cut

ends of the removed branches were greater than one meter from the intended measurement segment. In all cases the branches were relaxed (as in Wheeler et al. 2013) before sample excision. This was accomplished by covering the leaves with a plastic bag and sequentially cutting 2 cm increments off the base of the branch until 20-30 cm had been removed. The branch was then allowed to relax with the cut end under water for a minimum of thirty minutes. More dehydrated branches could require more time to reduce the xylem tension to less than 0.1MPa. To increase the range of xylem tensions sampled, several branches were dried in large plastic bags in the lab for up to two days. After a leaf was removed to measure xylem pressure these branches were relaxed in the same manner.

Vessel length measurements

Maximum vessel length was measured following Greenidge (1952). Due to the long maximum vessel length of *Robinia*, each segment was checked after excision to ensure that there were no vessels open from the measurement segment to the cut end of the branch. After this check twenty nine of the branches were cut back from the base until bubbles were seen indicating an open vessel.

Mean vessel lengths were determined by silicone injection following the protocol of Christman, Sperry & Alder (2009), with the calculation as modified by Jensen, Huggett & Holbrook (2013). Branches were flushed for 30 minutes prior to injection with a 20 mmol solution of potassium chloride in ultrafiltered de-ionized water (Millipore MilliQ UV plus) at a pressure of 0.1 MPa. A fluorescent dye (2,5-Bis (5-*tert*-butyl-benzoxazol-2-yl) thiophene; Sigma-Aldrich, Allentown, PA, USA) was added to the silicone mixture (RTV-141; Bluestar Silicones, York, SC, USA) prior to injection. The stems were

injected at 0.075 MPa overnight and then allowed to harden at room temperature for several days before they were sectioned.

Hydraulic parameters

Xylem tension was measured using a pressure chamber (Soil Moisture Equipment, Santa Barbra, CA). For all the samples collected using the lift, the leaves used to measure xylem tension were placed in foil wrapped plastic bags on the tree several hours prior to removal. For branches removed from the tree by pole pruners, leaves used to determine xylem tension were removed from the branches after a 30 minute equilibration period (prior to relaxation) during which the entire branch was sealed in a humid plastic bag. These samples will not indicate the xylem tension of the tree at the time the branch was excised, but they should indicate the minimum tension experienced by the branch during the collection process.

Stem hydraulic conductivity was measured as the flow rate driven through the stem by a known pressure gradient generated by a hydraulic head (Sperry, Donnelly & Tyree 1988) using an electronic balance (Sartorius CPA224S, Sartorius Corporation, Bohemia, NY). Leaf area distal to the conductivity segment was measured on a leaf area meter (LI-3100, Li-Cor, Lincoln, NE).

Stem vulnerability to embolism was estimated using the centrifuge method (Alder, Pockman, Sperry & Nuismer 1997). Stems were excised from relaxed branches. In all cases, except the native sap measurements, stems were flushed as above (20 mmol KCl in ultra-filtered water, flushed for 30 min at 0.1MPa) and the maximum conductivity measured (K_{\max}). Excised stems 0.14 m in length were placed in a custom made centrifuge rotor and spun to impose the desired tension. After each spin conductance was

measured (K_x). Percent loss conductivity (PLC) was calculated as $1 - K_x / K_{max}$ for each pressure. A weibull function was fit to each group of data by least squares regression.

Leaf turgor loss point was estimated from the inflection point of relationship between balancing pressure and leaf relative water content for 19 leaves following the protocol of Rockwell (2010).

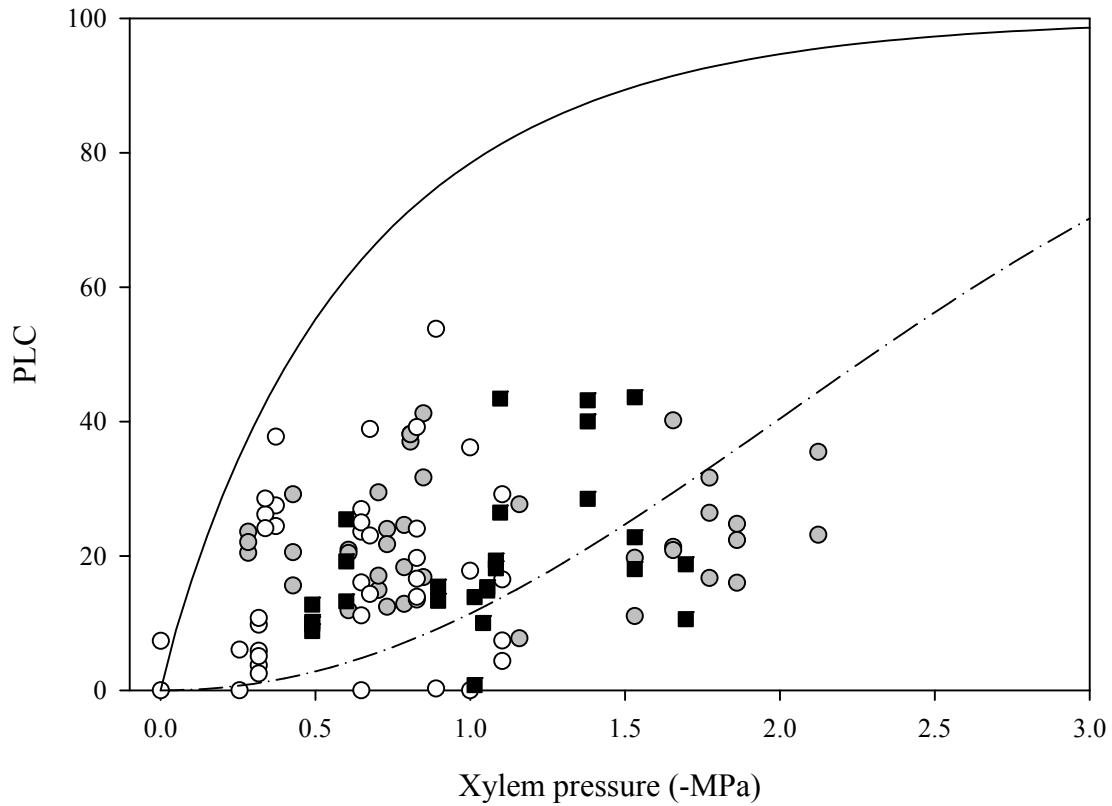
Statistics

For all statistical comparisons, PLC data were arcsine transformed and negative PLC values (which were always less than 10%) were set to zero (Sokal & Rohlf 1995). Negative PLC values are generally considered to arise when there is little native PLC and the flushing results in a nominal loss of conductivity (Wheeler et al. 2013). Statistical tests were performed in SPSS (IBM SPSS statistics version 20, 2011). Weibull curves were fit to the centrifuge PLC data in excel using its solver function and a modified spreadsheet designed to perform a least-squares regression for a two parameter Weibull function ($PLC = 1 - e^{-(xp/a)^b}$), where xp is induced xylem pressure and a and b are coefficients of the regression.

RESULTS

On tree PLC was highly variable throughout the collection period, with only a slight trend to increase after the mid-summer heat wave (Figure 4.1). The different methods of collecting resulted in significantly greater measured PLC in the dry collected samples ($p=.016$ independent samples T-test) over the range at which the later season vulnerability curves suggested little PLC should occur (between 0 and -1.2 MPa).

Figure 4.1



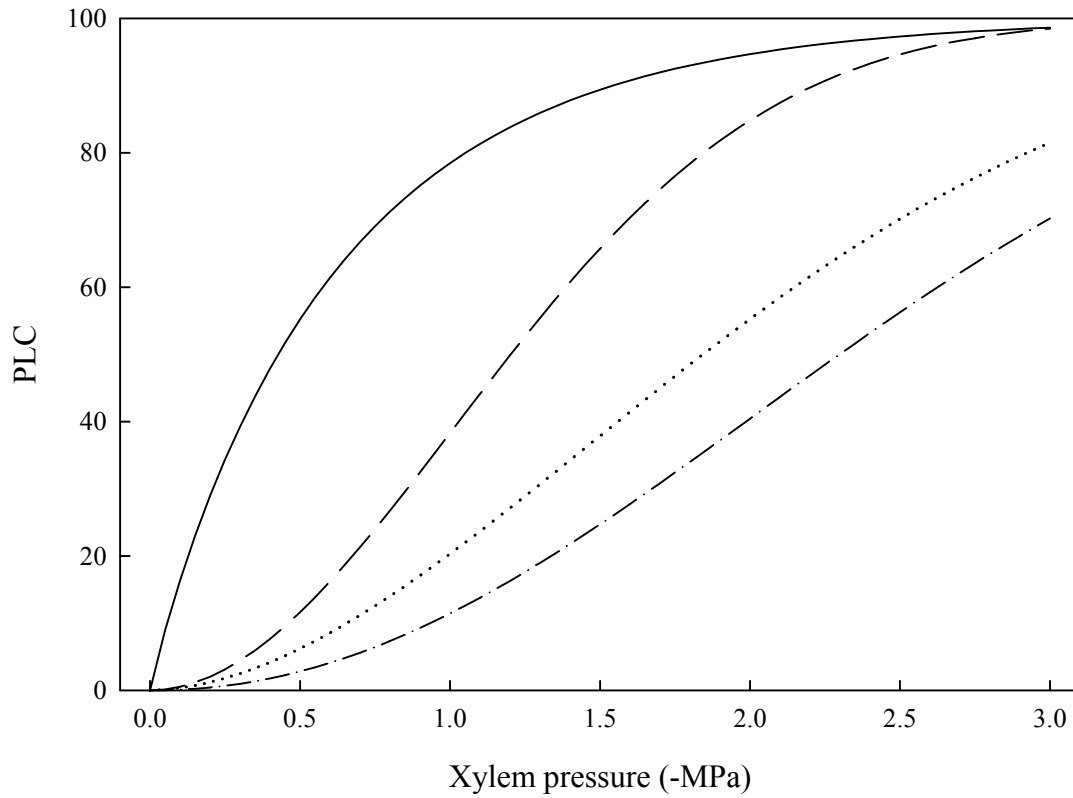
Percent loss of conductivity (PLC) in excised branches as a function of the measured xylem pressure prior to tension relaxation. Circles are branches collected in August after the heat wave. The white circles are branches collected wet and the gray circles are those collected dry. Black squares are from branches collected in June. The solid line is a Weibull function fit to the centrifuge curve data collected in June on multi-year old wood. The dash-dotted line line is a Weibull function fit to the centrifuge curve data collected in August using current year's growth.

The large variability in on tree PLC was reflected by equally large variability in the centrifuge derived vulnerability curves (Figures 4.2 and 4.3). The early season curves showed a pronounced “r” shape, with a P50 of -0.4 MPa (n = 9), which contrasted with more sigmoid to linear centrifuge curves from later in the season. Mid-season current year growth vulnerability curves had a P50 of -1.76 MPa (n = 9), mid-season two-year-old wood a P50 of -1.2 MPa (n = 5), and late season a P50 of -2.3 MPa (n=6). The native sap centrifuge measurements were more vulnerable than flushed centrifuge measurements for the same period at -1MPa ($p = 0.019$ one way ANOVA), likely a consequence of native embolism in the branches, but were not different at -2 MPa and -3 MPa (one way ANOVA, Figure 4.4, n= 6).

Despite the large variability in PLC and resistance to cavitation, the trees were quite proficient at avoiding xylem pressures that would induce PLC greater than 50%. On tree xylem pressure never became more negative than -1.1 MPa, which is less negative than the estimated leaf turgor loss point of -1.5 MPa to -1.7 MPa (Figures 4.1 and 4.5). Dried branches shed their leaves and dried leaves shed their leaflets at xylem tensions slightly more negative than -2 MPa, preventing measurements beyond this point (Figures 4.1 and 4.5).

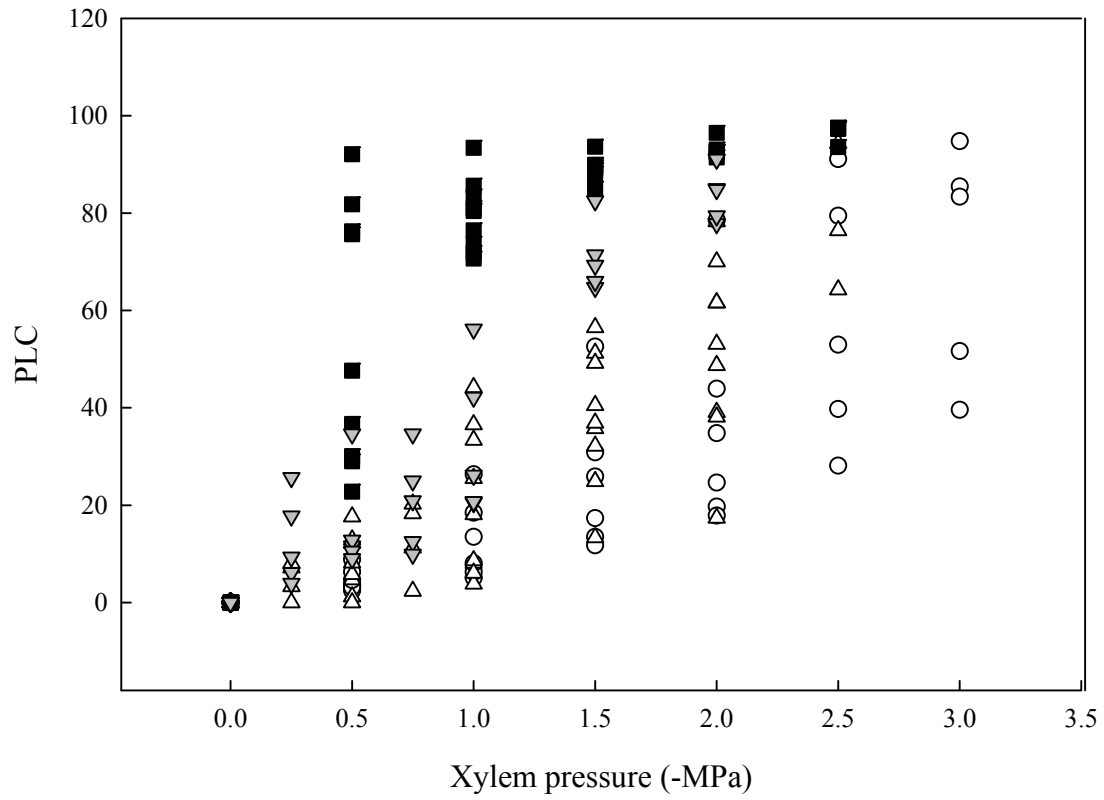
Vessel length also appeared to be highly variable (Figure 4.6), with maximum vessel length spanning a range from 0.26 m to 1.16 m, while the mean vessel length appeared to fall into two classes. The vessels found in lateral side shoots were shorter, with a mean length of 0.09 m and a standard deviation of 0.03 m (n=4 branches), while the vessels in the dominant leader had a mean length of 0.19 m and a standard deviation of 0.03 m (n=5 branches).

Figure 4.2



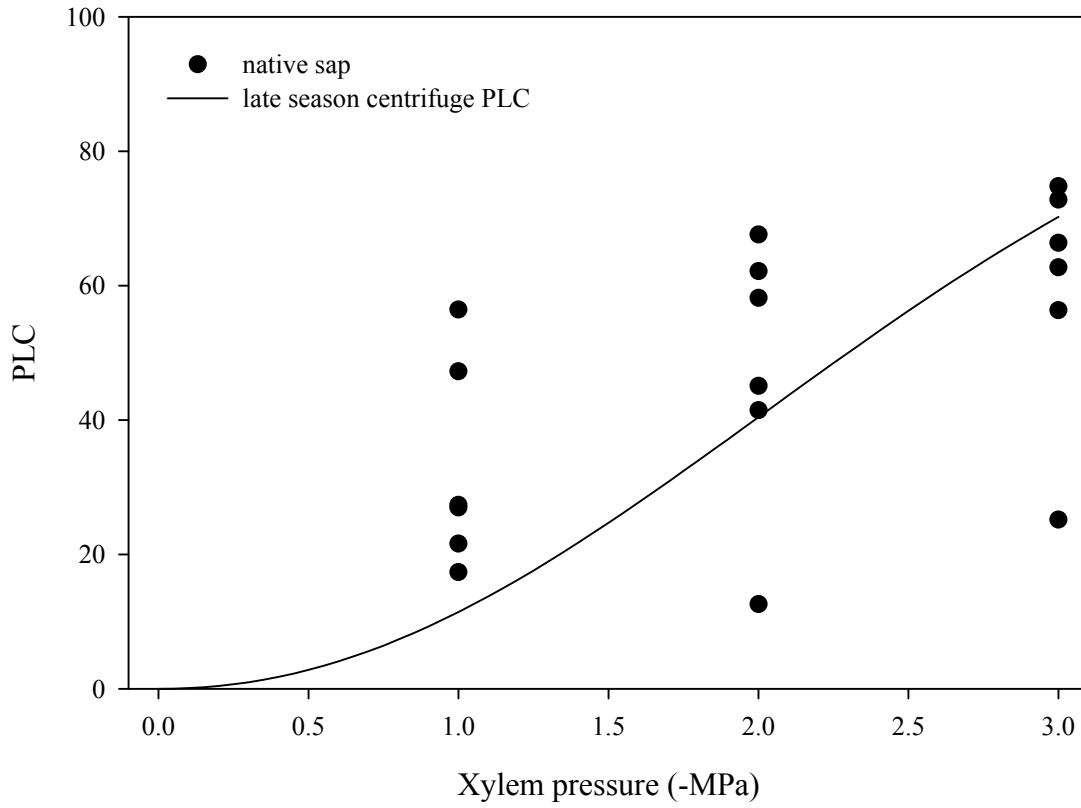
Weibull functions fit to centrifuge data by least-squares regression. The solid line is the vulnerability curve of early season multi year growth ($r^2 = 0.97$), the dotted line mid-summer current year growth ($r^2 = 0.87$); the dashed line mid-summer 2-year growth ($r^2 = 0.97$) and the dot-dash late summer lift data ($r^2 = 0.88$), which is current year growth.

Figure 4.3



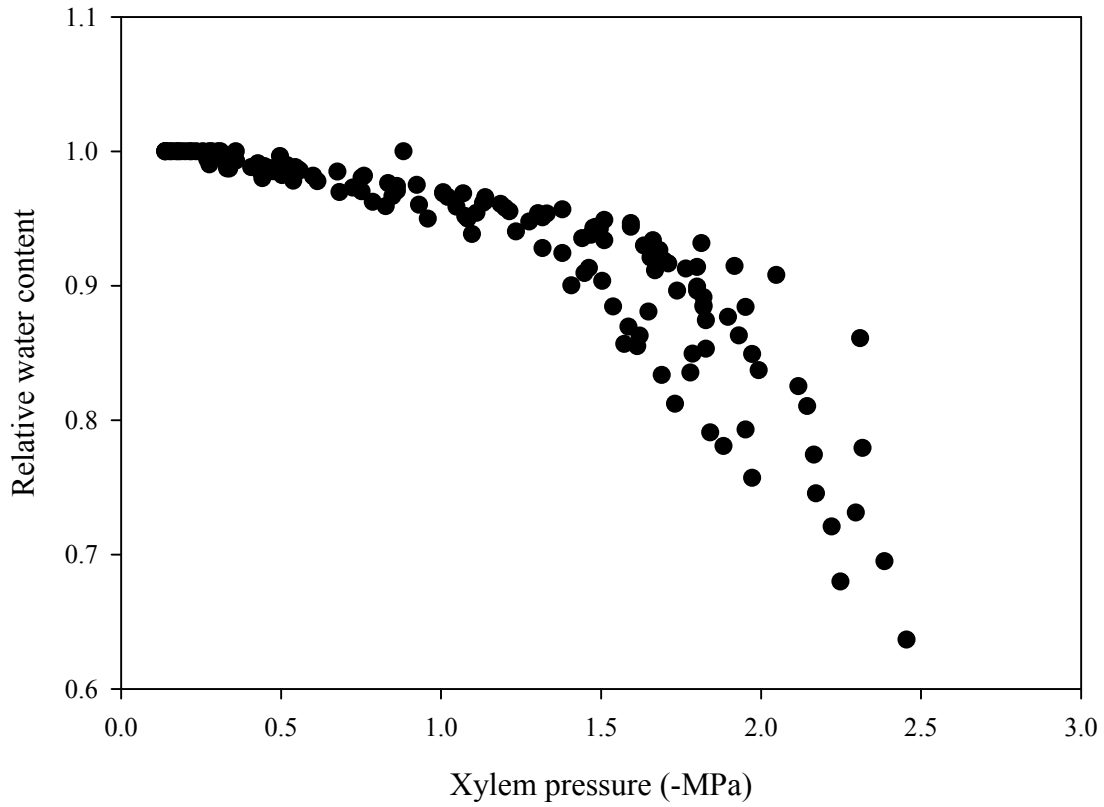
Individual PLC data for centrifuge vulnerability curves. Dark squares early season multi-year growth; open triangles mid-season current year growth; gray triangles mid-season 2-year growth; open circles late summer lift data.

Figure 4.4



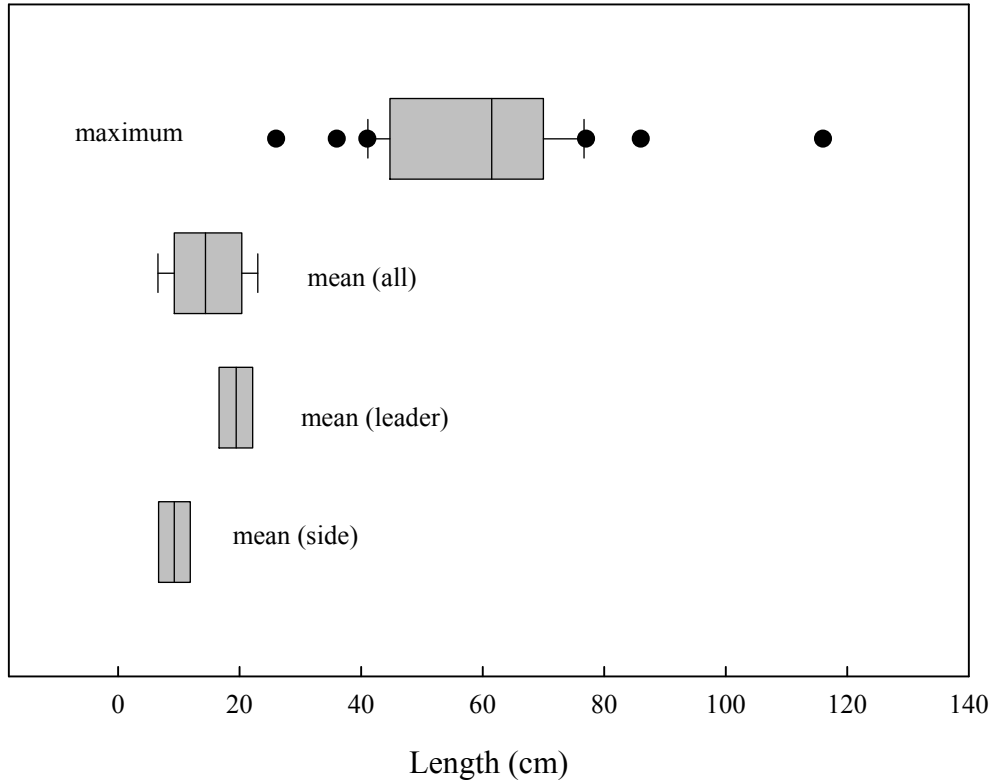
Individual PLC for native sap centrifuge measurements (black circles) and the late season centrifuge vulnerability curve (from samples taken within a week of the native sap measurements).

Figure 4.5



Relative water content as a function of the xylem pressure for the upper half of compound leaves. The turgor loss point is the inflection point of the relationship ranging from -1.5 MPa to -1.7 MPa.

Figure 4.6



Box plots of the mean and maximum vessel lengths. Mean vessel lengths were determined by silicone injection, maximum vessel lengths were determined by air. Mean (all) is the mean vessel length for all silicone injected segments, which are separated by category by their position on the sampled branches. The central bar for each plot is the median length; the boundaries of the box mark the 25th and 75th percentile and the whiskers the 10th and 90th percentiles.

DISCUSSION

Initial measurements of *Robinia* did indeed produce 'r' shaped vulnerability curves, but subsequent measurements tended to be more sigmoid to linear (Figure 4.2). Even the mid-season two-year-old wood vulnerability curve, while more vulnerable than the current year growth branches (supporting the idea that older xylem produces greater PLC in the centrifuge), did not exhibit substantial embolism until -1 MPa. It is unclear whether this early season 'r' shaped curve is a result of vulnerable xylem resulting from winter embolism, or a function of the older wood used in the measurements, which may have had longer vessels and been subject to artifactually generated embolism resulting from open vessels in the centrifuge.

While the on-tree measurements did indicate substantial and highly variable native embolism, this embolism did not exceed 50% and was consistent with the mid- and late-season centrifuge vulnerability curves (Figure 4.1). These curves also exhibited a high degree of variability (Figure 4.3), implying either that resistance to vulnerability within *Robinia* is quite variable, or that the variation in vessel length impacts the measured degree of embolism in the centrifuge. The highly variable degree of native embolism may have resulted in part from the summer heat wave, but the on-tree PLC data before this point still show substantial native embolism, especially at xylem tensions more negative than -1 MPa. This implies that *Robinia* may indeed operate with a substantial portion of its xylem embolized and simply have sufficient conducting capacity to sacrifice some portion to embolism.

The native sap centrifuge measurements were intended to provide insight into the potential open vessel artifact suggested to occur when long-vesseled species are

embolized in the centrifuge (Cochard et al. 2010). The native sap presumably does not contain micro-bubbles that can be introduced during flushing, and therefore if flushing contributes to artifactual embolism, native sap measurements should exhibit a lower degree of embolism than stems that are flushed prior to spinning. In *Robinia* the degree of background embolism is sufficiently high to obscure any trend that might exist for the -1 MPa spins, where this effect should be the most pronounced, making the data difficult to interpret. But the -2 MPa and -3 MPa data suggest that little embolism is occurring as a consequence of flushing.

The subtle, but significant, difference in measured PLC between wet and dry collected material (Figure 4.1) is dismaying because it indicates that despite the precautions of cutting branches with no open vessels between the measurement segment and the cut end, and relaxing the branches prior to sample excision, sample collection still appears to have an impact on measured PLC. This calls into question whether it is possible to accurately assess native embolism in long-vesseled species, and whether even the wet collected samples might be subject to some degree of collection induced embolism.

The large variability in mean vessel length between branches (Figure 4.6) and the fact that they fall into two classes may shed some light on the variability of both the centrifuge curves and the native embolism measurements. If long vessels are subject to a higher probability of artifactual induced cavitation (both in the centrifuge and on the tree due to cutting artifacts), then the variability of vessel length will be reflected in PLC measurements. It is worth noting that the bimodal distribution of mean vessel lengths is unusual and has not been reported previously. Generally, a single vessel length distribution is considered to represent all the distal organs of an individual, but here this

is demonstrated not to be the case. This may lead to substantial mischaracterization of a study species and over- or underestimates of the number of vessels open through a measurement sample, which can have consequences for the interpretation of PLC data and specific conductivity data.

The most salient consequence of the ‘r’ shaped curve debate is whether long-vesseled species operate with substantial native embolism or a water column that remains largely intact under most circumstances. This is not an esoteric debate. The difference in specific conductivity can vary three to four-fold depending on whether the xylem is water filled or air filled, and this can have significant consequences for whole tree transpiration. Also, substantial levels of native embolism can imply “novel refilling” (Taneda & Sperry 2008; Zufferey et al. 2011). The *Robinia* native embolism data, while ambiguous to some extent, do demonstrate that the trees avoid cavitation exceeding 50% of their conducting capacity and the trees’ behavior clearly demonstrates adaptations to avoid severe water stress. In situ xylem tension never became more negative than -1.1 MPa and when branches were removed from the tree and dried they shed their leaves at approximately -2 MPa, preventing further drying (Figure 4.1). These traits imply that *Robinia* is adapted to minimize embolism induced by water stress.

The use of centrifugal force to generate vulnerability curves has attained widespread use due to its convenience and speed, but whether the data from different species obtained by this method are directly comparable has rarely been addressed. The tension gradient exerted by centrifugation is parabolic in profile, with the minimum pressure achieved only at the center of the segment, while the ends remain in equilibrium with the atmosphere. For species with very short conduits, only those conduits crossing the center

of the segment will experience the tension against which PLC measurements are plotted. Until substantial embolism occurs and the resistance of the central portion of the stem becomes dominant, centrifuge curves for these species will underestimate the degree of embolism present (Cochard, Damour, Bodet, Tharwat, Poirier & Améglio 2005). Conversely, for long vesseled species, most conduits will cross this central point of minimum pressure, resulting in a more uniform impact of embolism. However, this embolism will likely be inflated by artifactually induced embolism resulting from air bubbles in vessels open from the cut end that extend into the measurement segment. Once the fluid in these vessels is under centrifuge induced tension any air bubbles not impeded by pit membranes will “float” to the central portion of the rotating stem due to the gradient in pressure. These bubbles can then induce cavitation that would not have occurred if the vessel were not open to the cut surface. These two competing mechanisms may result in an optimum vessel length for measurement in the centrifuge, where vessels are long enough to not be subject to underestimation of embolism due to insufficient overlap from the central portion, but not too long so as to limit the degree of bubble penetration from the cut ends to the central low pressure portion of the segment. The vulnerability of species whose vessel-lengths fall outside this optimum range may not be directly comparable using the centrifuge. In terms of centrifugation artifacts, *Robinia* should fall into the too long category based upon the mean vessel length, but further measurements are necessary to evaluate the degree to which open vessels might inflate the PLC measured by centrifugation.

These potential centrifuge artifacts stand in stark contrast to bench-top dehydration, which if performed correctly imposes a uniform tension on all the closed vessels within

the treatment branch, eliminating the potential for both types of artifacts. However, the variability of native PLC data in this study and the difference in PLC data between wet and dry collection methods are cautionary regarding the ease with which a bench-top dehydration vulnerability curve can be measured on a species with long vessels.

The original goal of this study was to determine whether ‘r’ shaped vulnerability curves measured on long-vesseled species by the centrifuge technique accurately reflect the species’ vulnerability to embolism, and if cutting artifacts could inflate measured native embolism implying that the centrifuge vulnerability curves are correct. Centrifuge curves measured on multiple-year growth early in the season do appear artificially vulnerable, but those measured on current year growth are consistent with native embolism data, implying that the difficulty may not be with long vessels so much as with long vessels that have been embolized prior to flushing and measurement. The high degree of variation both in native PLC measurements and in centrifuge generated vulnerability curves remains unexplained. This may indeed be a function of cutting artifacts and an open vessel artifact. A further experiment visualizing which vessels are embolized and if open vessels have a higher probability of embolism would provide insight into whether open vessels are artificially vulnerable when measured in the centrifuge.

ACKNOWLEDGEMENTS

We would like to thank Andrew Knoll for his helpful comments on this manuscript.

Chapter 5

Investigating xylem embolism formation, refilling and water storage in tree trunks using frequency domain reflectometry. *

ABSTRACT

Trunks of large trees play an important role in whole-plant water balance but technical difficulties have limited most hydraulic research to small stems, leaves and roots. To investigate the dynamics of water-related processes in tree trunks, such as winter embolism refilling, xylem hydraulic vulnerability, and water storage, volumetric water content (VWC) in the main stem was monitored continuously using frequency domain moisture sensors in adult *Betula papyrifera* trees from early spring through the beginning of winter. An air injection technique was developed to estimate hydraulic vulnerability of the trunk xylem. Trunk VWC increased in early spring and again in autumn concurrent with root pressure during both seasons. Diurnal fluctuations and a gradual decrease in trunk VWC through the growing season were observed, which, in combination with VWC increase after significant rainfall events and depletion during periods of high water demand, indicate the importance of stem water storage in both short-and long-term water

* reprinted from: Hao G.¹, Wheeler J. K.¹, Holbrook N. M. & Goldstein G. (2013) Investigating xylem embolism formation, refilling and water storage in tree trunks using frequency domain reflectometry. *Journal of experimental botany* **64**, 2321-2332.

1: Authors contributed equally

balance. Comparisons between the trunk air injection results and conventional branch hydraulic vulnerability curves showed no evidence of “vulnerability segmentation” between the main stem and small branches in *B. papyrifera*. Measurements of VWC following air injection indicate that embolized vessels can be refilled by active root pressure but not in the absence of root pressure. The precise, continuous and non-destructive measurement of wood water content using frequency domain sensors provides an ideal way to probe many hydraulic processes in large tree trunks that are otherwise difficult to investigate.

INTRODUCTION

The tree trunk, with both long distance water transport and storage functions, plays an important role in whole-plant water relations (Goldstein *et al.* 1998; James *et al.* 2003). However, due to technical difficulties most studies on tree hydraulic architecture are limited to peripheral organs, such as roots, terminal branches and leaves, while studies of trunks of adult trees are largely absent. A long-held hypothesis regarding the spatial patterns of plant hydraulics along the whole-plant water transport pathway is known as hydraulic segmentation (Zimmermann 1978; Zimmermann 1983). According to this paradigm, plants have structural features that prioritize the water flow continuity in the main axis to the terminal apex. Tyree and Ewers (1991) advanced Zimmermann’s segmentation theory by adding the “vulnerability segmentation” hypothesis. They argued that both higher hydraulic conductivity and stronger resistance to drought-induced cavitation might be necessary for a tree to maintain an intact water column in its main stem at the expense of its peripheral organs; however, few studies have examined the cavitation vulnerability of big stems or trunks of large trees. In the present study a trunk air injection technique was developed to estimate the xylem vulnerability to air seeding in

main stems of adult *Betula papyrifera* Marsh, which is a deciduous tree with diffusively distributed vessels in the xylem.

Besides drought, freezing-thaw event is another important factor that can induce substantial cavitation in temperate trees during cold seasons (Magnani & Borghetti 1995; Jaquish & Ewers 2001; Améglio, Bodet, Lacoïnte & Cochard 2002). When the xylem sap freezes, dissolved gases come out of solution and form bubbles that can nucleate cavitation upon thawing when tensions are reestablished (Hammel 1967; Zimmermann 1983). According to Laplace's law, bubbles smaller than a critical size do not expand and re-dissolve easily, while larger bubbles tend to expand under tension (Oertli 1971; Vogel 1988; Yang & Tyree 1992). Therefore, larger-volume xylem conduits are more vulnerable to freeze-thaw induced embolism than smaller-volume ones because they contain more dissolved gas and form larger bubbles during freezing (Ewers 1985; Sperry & Sullivan 1992). Embolism development over the winter caused by freeze-thaw cycles can be extensive in branches of temperate trees, in some cases eliminating all hydraulic conductivity by the end of the winter (e.g. Cochard & Tyree 1990; Sperry & Sullivan 1992; Wang *et al.* 1992). Tree trunks with larger xylem conduits compared to those of smaller branches may also develop extensive cavitation over the winter although this has not been documented except in saplings (Sperry *et al.* 1988).

Cavitation does not pose a serious problem during winter, but may impair growth during the following season if newly produced xylem alone is insufficient to supply water to the crown. In many species, mechanisms have evolved for restoring hydraulic conductivity in the spring by refilling the embolized vessels using positive root pressure (Sperry *et al.* 1987; Sperry *et al.* 1994; Hacke & Sauter 1996; Améglio *et al.* 2002; Cobb,

Choat & Holbrook 2007). Although root pressure has been well documented in many temperate tree or vines species for winter embolism refilling, the dynamic change of xylem water status during the repair process has not been examined, especially in the main stems of adult trees.

The root pressure (RP) and trunk volumetric water content (VWC) were measured during the spring throughout the embolism refilling process in *B. papyrifera*. To further investigate the dynamics of trunk water status pertaining to whole-plant water balance, VWC was measured continuously through the summer season to the end of the year until freezing occurred in the xylem. The non-destructive and continuous *in situ* measurements of trunk wood water status permits us to investigate in detail a series of important questions regarding plant water relations, such as the role of trunk water storage in short (diurnal) and long term (seasonal) water balances, and embolism formation and repair.

Both the trunk xylem hydraulic vulnerability estimation technique and the continuous monitoring of trunk water status used in the present study are based on the novel use of frequency domain reflectometry (FDR) moisture sensors for the *in situ* non-destructive measurement of wood VWC in functioning trees. Moisture sensors based on the FDR technique have been widely used in measuring soil water content (Czarnomski, Moore, Pypker, Licata & Bond 2005), but have not been used in wood of living trees. The FDR moisture sensor, with high precision, high temporal resolution, and convenient installation configuration, is a potentially ideal way to measure stem water content in trunks accurately and continuously, providing insight into both short-and long-term hydraulic related plant physiological processes. Specifically, in the present study the simultaneous and continuous measurements of root pressure and trunk water content in

the spring provide information regarding the dynamics of winter cavitation refilling in the main stems of large trees.

MATERIALS AND METHODS

Study site and plant materials

The study was conducted at Harvard Forest (42° 32'N, 72° 11'W), Petersham, Massachusetts, USA. The study sites are mixed secondary deciduous forest stands, dominated by red oak (*Quercus rubra* L.) and red maple (*Acer rubrum* L.), with paper birch (*Betula papyrifera* Marsh.) commonly found. Average annual precipitation at this site is ca. 1000 mm and is distributed fairly evenly throughout the year. Annual mean temperature is 6.5°C, with the highest and lowest monthly mean temperatures occur in July (20°C) and January (-7°C), respectively. Adult *B. papyrifera* trees with diameter at breast height (DBH) ranging from 14.1 to 26.3 cm were used for the present study.

Volumetric water content

Moisture sensors based on frequency domain reflectometry (FDR) technology (Model GS3, Decagon Devices, Inc. Pullman, WA) were installed in the trunks of adult *B. papyrifera* trees at breast height. Three holes of 5.5 cm in depth spaced 2.54 cm apart were drilled radially into the trunk using an electric drill, with a drill bit of 3.26 mm in diameter. A customized drill guide with three holes of the same diameter was used to facilitate the drilling. After inserting the three prongs (5.5 cm in length, 3.26 mm in diameter and 2.54 cm apart) of the sensor into the drilled holes, the moisture sensors were gently hammered until the prongs were completely within the tree (Appendix Figure B.1). The gaps between the sensor overmold and the tree were sealed with silicon caulk to prevent infiltration of water from precipitation. The whole sensor was insulated with

foam and aluminum foil to avoid heating by direct sun light. In addition to VWC, trunk temperature was measured by the thermocouples embedded in the sensor overmold. Data were logged using EM50 data loggers (Decagon Devices, Inc. Pullman, WA) every 5 minutes for 24 hours a day. Three sensors were installed in three trees (numbered 1, 2 and 3) in early spring (March) of 2012 and measurements were made continuously until the end of the year. The FDR sensor calibration was conducted in the laboratory using wood of *B. papyrifera* and the results showed that the sensor VWC output based on the factory calibration matches well with VWC calculated by a gravitational method (Supplementary Appendix Figure B.2). The factory calibration was thus used in the present study.

Root pressure

Hydrostatic pressures in trunks of the same three trees were measured using electronic pressure transducers (PX26-015GV, Omega Engineering, Inc. Stamford, CT). Two holes of 1.59 mm in diameter and ca. 2 cm in depth were drilled near the base of a tree trunk and hypodermic needles of the same outer diameter with bent tips (to avoid clogging by wood debris) were inserted to the sapwood and glued in place with epoxy. The pressure transducers were connected to the hypodermic needles through a short piece of non-elastic water-filled tubing (Bev-A-Line IV, Cole Parmer Instrument Co., Chicago, IL). The output voltage of the pressure transducers was logged at 5-min intervals with a CR10X data logger (Campbell Scientific, Logan, UT, USA). Every two to three weeks, transducers were moved to fresh-drilled holes to avoid gradual clogging of xylem. Measurements of root pressure were made from March to June and from September to November 2012.

The pressure transducers were calibrated in laboratory prior to and after the field measurements and no significant drift was found. Briefly, transducers were connected with a high-precision digital pressure gauge (Model DPG1000AD; Omega Engineering, Inc., Stamford, CT) and were connected to a high-pressure nitrogen tank through a regulator. The pressure applied to the transducer was increased in a stepwise manner from 0 to ca. 138 kPa with an interval of 1 psi (ca. 6.89 kPa) and the output data were recorded using a CR10X data logger. Linear regressions between voltage outputs and applied pressures were used to calculate the root pressures.

Vulnerability to cavitation in tree trunks and terminal branches

To determine whether the early spring stem water content increase is directly related to the refilling of winter-embolized vessels, an air injection technique was developed to induce embolism in the sapwood where the moisture sensors were located. A high-pressure-resistant PEEK tubing (MicroSolv, Eatontown, NJ) was inserted into a freshly drilled hole in the tree trunk (ca. 1.59 mm in diameter and ca. 5 cm in depth) 2 cm above the installed FDR sensor and held in place with a customized tubing holder, which was tied to the trunk using a ratcheting tie-down (Appendix Figure B.1). The tubing was connected to a DPG1000AD digital pressure gauge (Omega Engineering, Inc.) and a high-pressure nitrogen tank through a regulator. An o-ring was placed around the PEEK tubing between the tree bark and the tubing holder to make a tight seal. The injection pressure was increased in a stepwise manner from 0 to 3.5 MPa at intervals of about 0.3 MPa. The pressure for air injection was kept constant at each level for 15-25 min until VWC (FDR sensor readings were continuously monitored with a computer) reached a steady state. Air injection was conducted on four trees (trees 2 and 3, plus two trees not

used for long-term VWC monitoring) in mid-April of 2012 before leaf flush when VWC had increased to a relatively stable level. To test whether embolism can refill in the absence of root pressure, injection was also conducted on another four trees (trees 5-7 and another tree not used for long-term VWC monitoring) at a different site in early June. Trees 1 and 4, which were not air-injected, were used as controls for spring and summer measurements, respectively. Continuous measurements of trunk water content were recorded for trees 4-6 (tree 7 was cut down after air injection) from 25 May 2012 to the end of the year.

Conventional hydraulic vulnerability curves were constructed using centrifugal force method on five small stem segments from different trees with diameters ranging from 0.5 to 0.8 cm (Alder *et al.*, 1997). Briefly, vulnerability to drought-induced cavitation was measured as the decrease of hydraulic conductivity (K_h) in response to a stepwise increase in xylem tension generated by spinning a 14.2 cm stem segment using a Sorvall high speed centrifuge (Model RC-5C Plus, Du Pont Instruments, Wilmington, DE) with a custom rotor (Alder *et al.*, 1997). The percentage loss of conductivity (PLC) following each spinning was calculated as $PLC = 100[(K_{max} - K_h)/K_{max}]$, where K_h is the hydraulic conductivity after each spinning and K_{max} is the maximum conductivity measured on flushed segments.

Dye perfusion

In June, one tree was cut down 0.9 m above the ground after the completion of air injection and a trunk segment ca. 60 cm (with the injection point in the middle) of the trunk was cut off. The segment was kept under water in a cooler and transported to the laboratory. Five centimeters were removed from both ends using an electric saw and the

remaining segment was connected to a soft polythene tube (modified from a ziplock bag) with a diameter of ca. 15 cm. Parafilm was used to ensure a tight seal between the tube and the segment. The segment was placed vertically and the reservoir created by the polythene tube on top of the stem segment was filled with 2 L of filtered 0.1% Toluidine Blue solution. On the second day, after all the staining solution in the reservoir had flowed through the trunk segment, it was cut transversely into two pieces in the middle to evaluate the effect of air injection on xylem hydraulic conductivity.

Wood anatomy

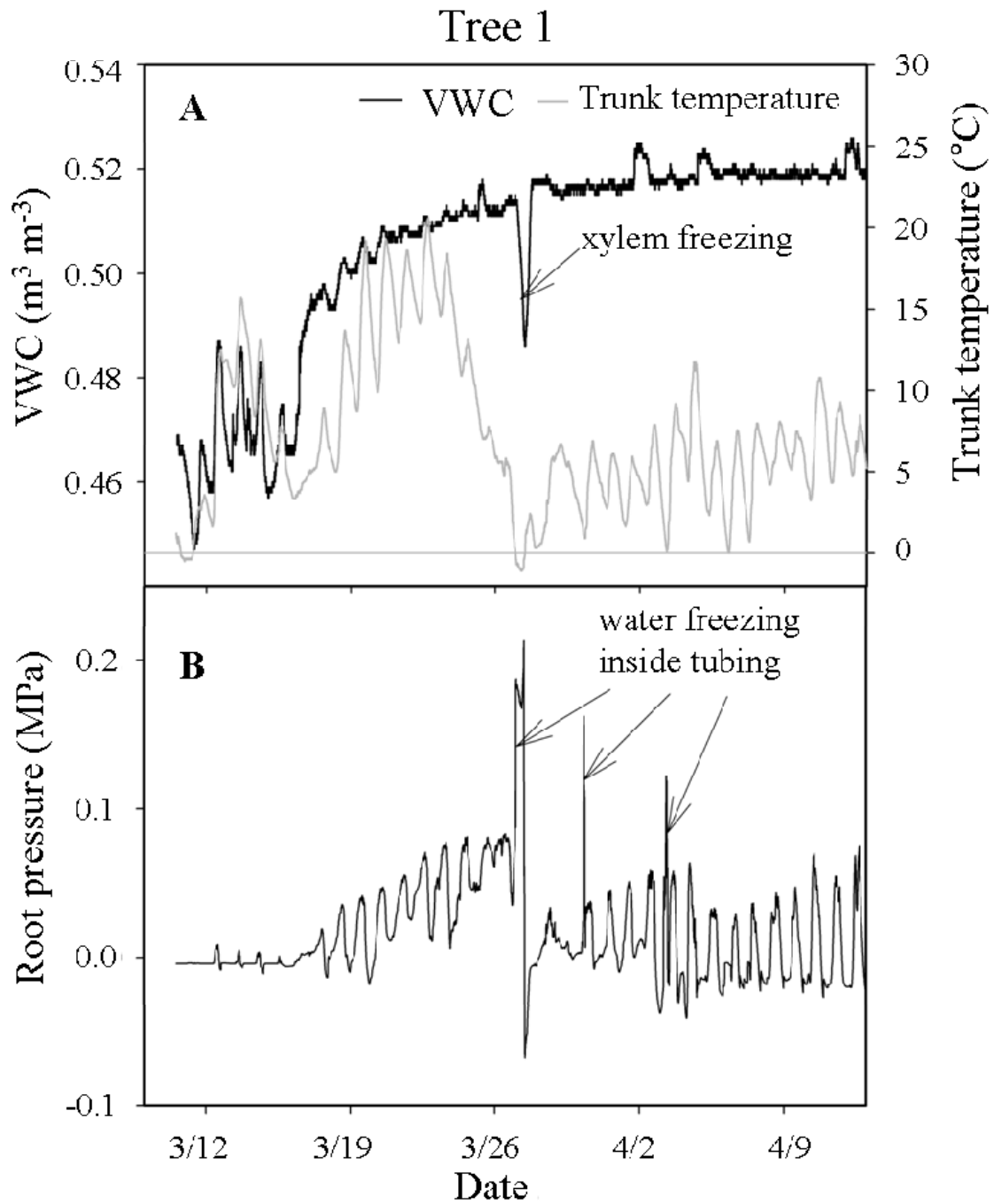
Small pieces of trunk sapwood were taken using a chisel from four trees at breast height and transverse sections of these wood samples were prepared using a sliding microtome (Reichert, Vienna, Austria). Wood cross-sections were also made on small branches with diameters ranging from 0.5-0.8 cm. These cross-sections were stained in a 0.1% Toluidine Blue solution and images were taken under 40× (trunk wood) or 100× (branch) using a digital camera (AxioCam HRC, Carl Zeiss, Jena, Germany) mounted on a light microscope (Olympus BH-2, Olympus, Tokyo, Japan). Vessel diameters were calculated using ImageJ software (US National Institutes of Health, Bethesda, MD) based on individual lumen areas assuming circular shapes.

RESULTS

Xylem water recharge under root pressure in early spring

With the occurrence of root pressure in mid-March 2012, trunk VWC quickly increased to a substantially higher level, which was on average $0.08 \text{ m}^3 \text{ m}^{-3}$ higher than the initial average value (Figure 5.1A and 5.1B). Before wood VWC reached a stable level, it exhibited regular diurnal fluctuations with higher values observed at midday, which

Figure 5.1



(A) Diurnal fluctuation and an overall increase in volumetric water content (VWC) in the trunk of a representative *B. papyrifera* tree during early spring; (B) root (xylem) pressure measured in the same trunk. The horizontal line in panel A shows tree trunk temperature of 0°C .

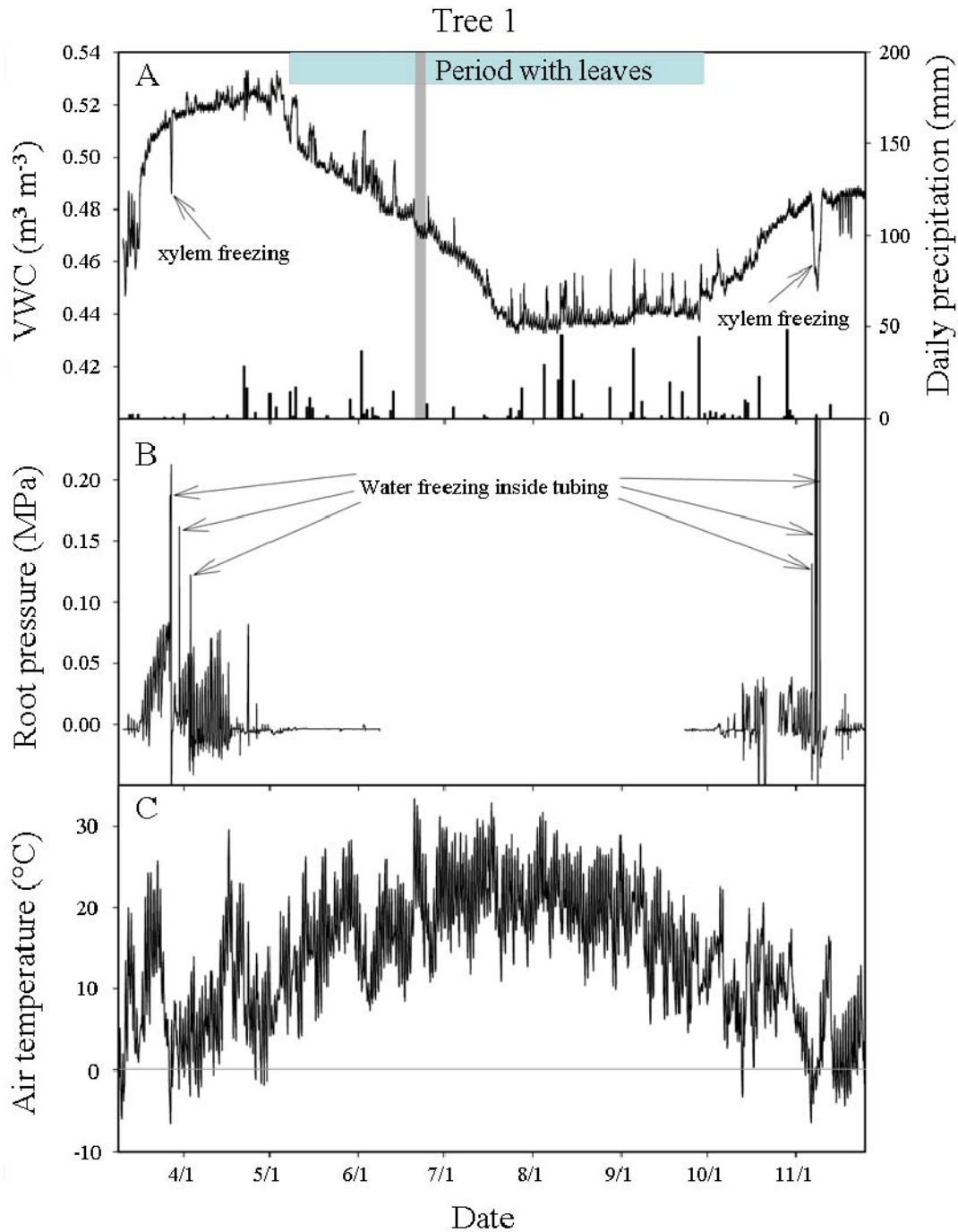
corresponded very well with the diurnal fluctuations in root pressure. After a maximum VWC level was reached, the diurnal fluctuation became negligible although root pressure continued to show a diurnal pattern of variation (Figure 5.1; Appendix Figure B.3 A).

The high resolution of the GS3 moisture sensor made it possible to measure subtle changes in wood water contents. However, when the water in the xylem froze the output of the sensor did not reflect the actual water content (Figures 5.1 and 5.2) due to the large difference in dielectric constant between liquid and solid water (80 vs. 3.2 for water and ice at 0 °C), which is the basis for calculating VWC (Kizito *et al.*, 2008). Thus in a few cases, such as on 27 March, the dramatic decrease in VWC readings from the sensor resulted from freezing rather than a change of absolute water content (including ice) in the trunk wood (Figure 5.1A). Likewise, the very high readings of pressure transducers during the few days with below freezing temperatures did not imply high root pressure but rather a pressure increase caused by the volume expansion of water during freezing in the tubes connecting the xylem and inside the transducer chambers (Figure 5.1B). Aside from these few days when freezing occurred, both VWC and root pressure measurements were very reliable.

Short-term and seasonal variations in trunk water content

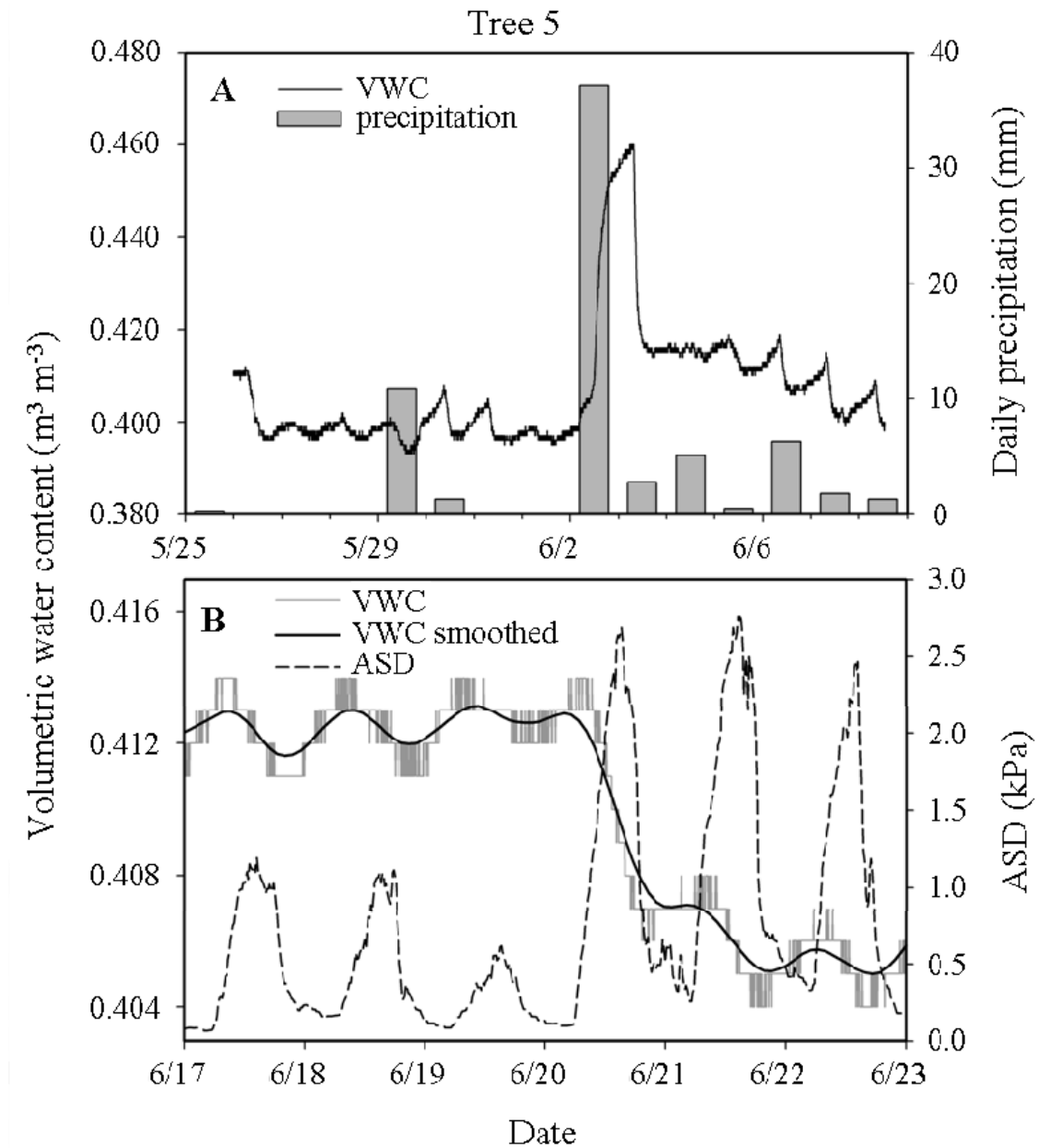
After full leaf expansion, diurnal fluctuations in trunk VWC reoccurred but with a different pattern compared to that found before and during xylem refilling; on sunny days, the maximum VWC occurred at night and the minimum values occurred around midday, which was consistent with the diurnal pattern of evaporative demand (Appendix Figure B.3C). Significant increases in trunk VWC during days with significant rainfall and gradual decreases thereafter were observed (Figure 5.3A; Appendix Figure B.4 and

Figure 5.2



(A) Seasonal change of trunk volumetric water content (VWC) in a representative *B. papyrifera* tree (thin line) and daily sums of precipitation (columns); (B) root pressure recorded in the spring and autumn of 2012; (C) environmental air temperature. The dark bar in panel A denotes the timing of the heat wave shown in Figure 5.3B. The horizontal line in panel C marks air temperature of 0 $^{\circ}\text{C}$.

Figure 5.3



(A) Recharge of trunk volumetric water content (VWC) in a representative tree during a significant rainfall event on 2 June 2012; (B) substantial decrease in trunk VWC due to a heat wave occurred in the early summer, during which air saturation deficit (ASD) of the atmosphere (Fisher Meteorological Station of Harvard Forest) was substantially higher than normal.

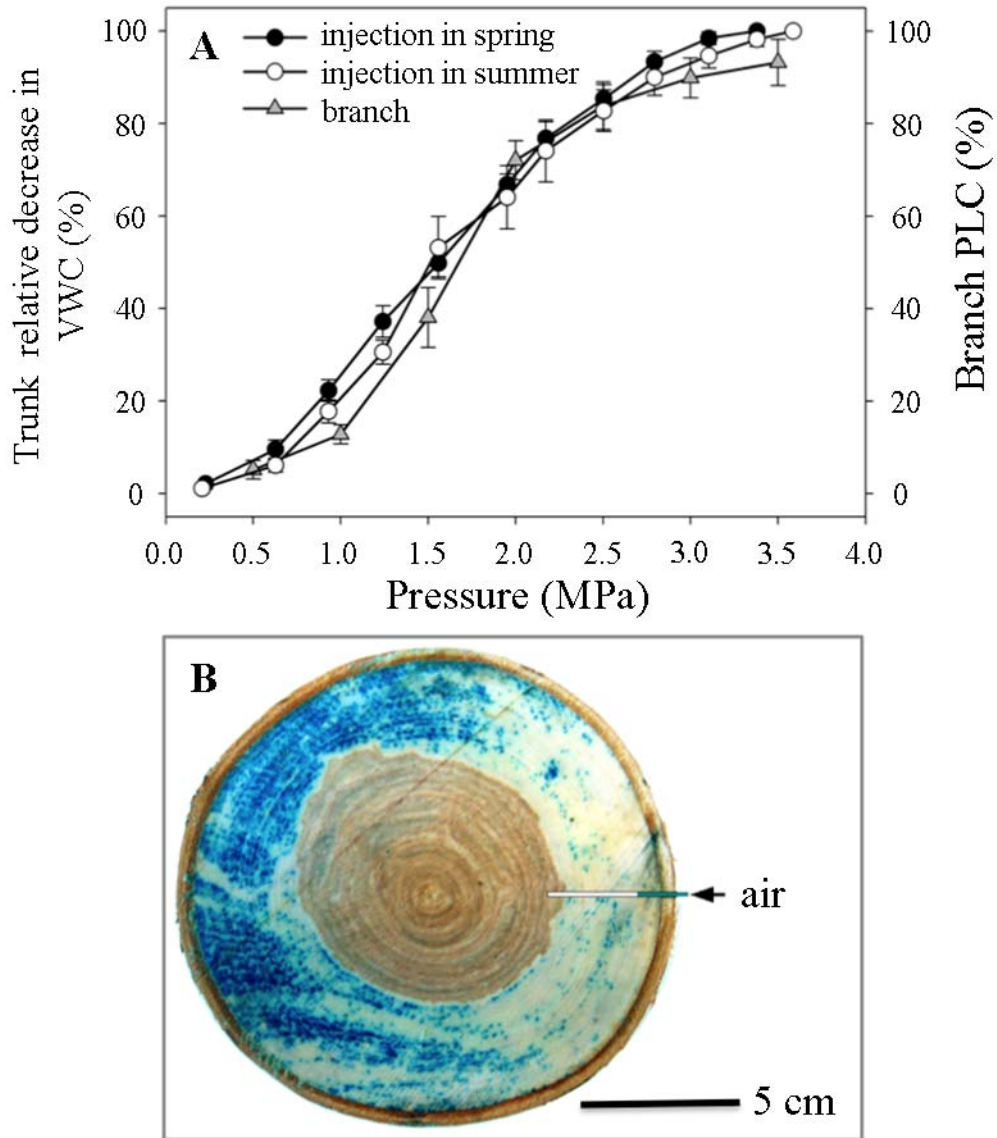
Figure B.5). During a heat wave in late June, VWC of the trunk decreased substantially indicating the use of internal water storage in the trunk to buffer high canopy water demands due to elevated air saturation deficits (Figure 5.3B; Appendix Figure B.4 and B.5).

After leaf expansion (around 30 April), root pressure diminished and the overall VWC in the trunk started to decrease gradually until the end of July (Figure 5.2A and 5.2B; Appendix Figure B.4 and B.5). Trunk VWC started to increase again in the autumn and more noticeably with the resumption of root pressure in October and early November (Figure 5.3A and 5.3B; Appendix Figure B.4 and B.5). Note that the VWC increase in the autumn occurred over a period of about one month and was not as dramatic as that occurred in the early spring, which rapidly reached a maximum level over roughly two weeks from the initiation of root pressure (Figure 5.1A; Appendix Figure B.4 and B.5).

Cavitation induced by air injection and recovery under root pressure

The injection of air into the tree trunk close to the FDR sensor resulted in a pattern of VWC reduction similar to the decline in hydraulic conductance observed for stems subjected to air injection or centrifugation. When the change of VWC in response to each air injection was expressed relative to the total VWC change observed at the highest applied pressure, the curves are similar to hydraulic vulnerability curves measured on small stem segments of the same species (Figure 5.4A). At a pressure of 1.6 MPa, a 50% of the relative VWC decrease was obtained that is not significantly different from the centrifugal force causing 50% loss of hydraulic conductivity in small stem segments (1.7 MPa; $P > 0.05$, t -test). The dye perfusion result showed that air injection resulted in a

Figure 5.4



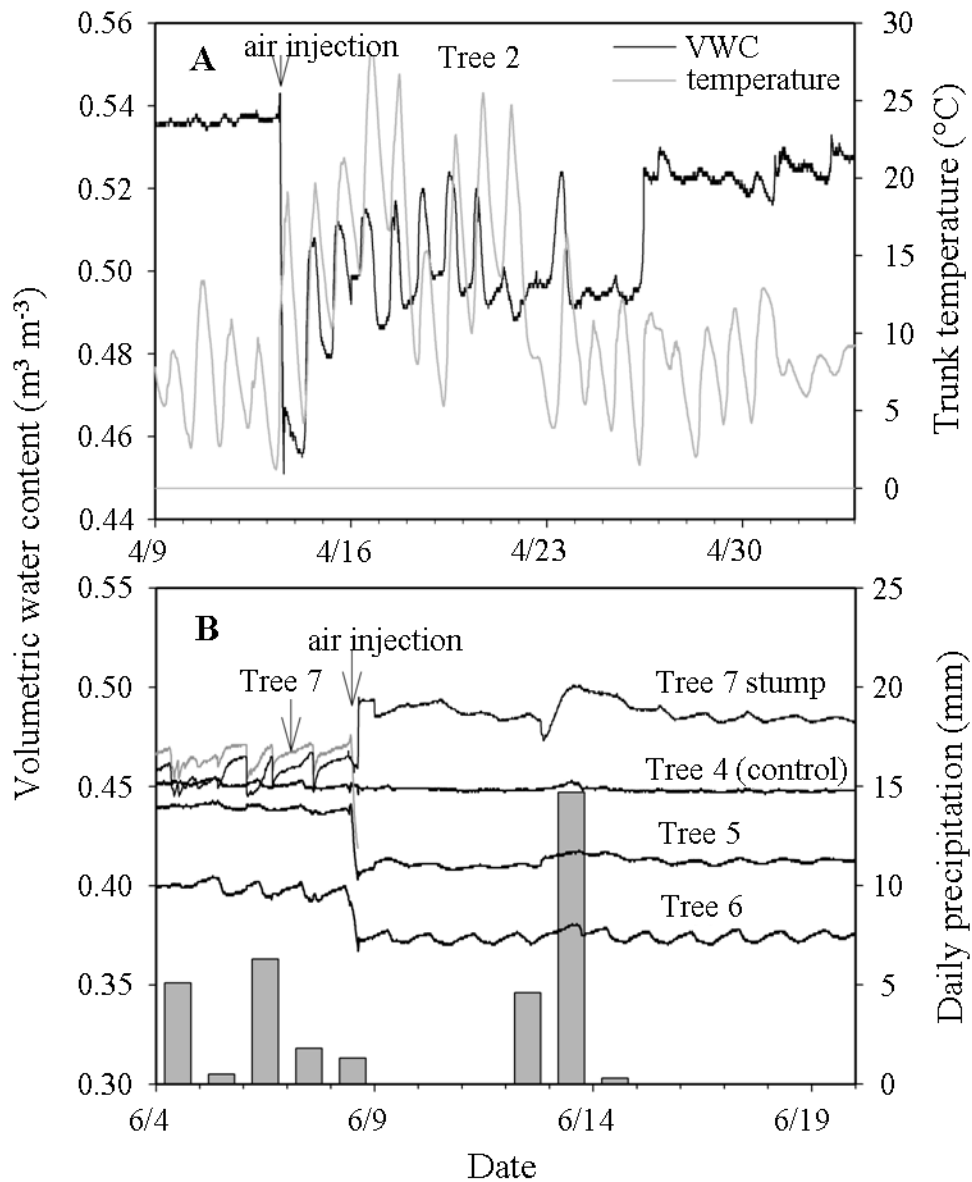
(A) Percentage change in VWC (relative to the total VWC decrease achieved at highest applied air pressure in the trunk) versus injection pressure and percentage loss of conductivity (PLC) as a function of centrifugal force measured on small branches. Error bars show ± 1 SE ($n = 4$ for trunk VWC in both spring and summer and $n = 5$ for branch PLC); (B) results of dye perfusion showing that the xylem close to the injection site became cavitated heavily after air injection.

large number of air filled non-conductive vessels in the xylem close to the injection point (Figure 5.4B).

In trees that were still generating root pressure (prior to leaf flush), the post-air-injection VWC gradually increased before it decreased again due to leaf expansion (Figure 5.5A; Appendix Figure B.4A and B.4B). Notably, after air injection obvious diurnal fluctuations in VWC reoccurred (Figure 5.5A; Appendix Figure B.4A and B.4B) in a pattern similar to those naturally occurred in March (Appendix Figure B.3A), with higher values observed at daytime.

Air injection in early June, after leaves were fully expanded, also resulted in significant decrease in VWC but with a smaller magnitude relative to the injection before leaf flush (0.05 vs. $0.11 \text{ m}^3 \text{ m}^{-3}$), which may resulted from an overall lower pre-injection VWC in June than in April. The large variation among trees in the pre-injection VWC (0.50 to $0.54 \text{ m}^3 \text{ m}^{-3}$ in the spring and 0.38 to $0.48 \text{ m}^3 \text{ m}^{-3}$ in the summer) may be also in part due to differences in sapwood depth and site soil water availability (the spring site located along a brook and the summer site located on a hill slope). These factors may have resulted in large differences in the absolute VWC change among trees during air injection. This problem may be partially solved by using shorter FDR probes in the future. For this reason the VWC change was expressed in a relative term, i.e. decrease in VWC under each pressure relative to the maximum VWC decrease under the highest applied pressure. In the freely transpiring trees, no significant recovery in VWC was observed (Figure 5.5B), indicating a lack of xylem refilling in the absence of root pressure. In the stump of the tree that was cut down after air injection, VWC rapidly increased to a significantly higher level within an hour (Figure 5.5B), reflecting the disappearance of xylem tension.

Figure 5.5



(A) Decrease of trunk volumetric water content (VWC) due to air injection and its gradual recovery under root pressure in early spring in a representative tree; (B) measurement of VWC following air injection conducted in early June in the absence of root pressure showing no significant recovery of VWC in transpiring trees. Two sensors were installed in tree 7; one at ca. 70 cm above the ground (data shown as 'tree 7 stump') and another at breast height, which was removed when the tree was cut down after air injection (the gray line shown as 'tree 7').

Xylem anatomy

Transverse sections showed that in the trunk xylem of *B. papyrifera* about 15% of the whole cross-sectional area is occupied by the lumens of diffusely distributed vessels and most other area is filled with fibers, with no more than 10% of the cross section occupied by thin lines of ray parenchyma (Appendix Figure B.6). Average vessel diameter in trunk sapwood is much larger than in small branches (95.5 vs. 32.7 μm ; $P < 0.001$).

DISCUSSION

Embolism repair under root pressure in early spring

The trunk wood VWC data, in this study, showed that it took about two weeks from the first day of root pressure formation until the VWC reached stable maximum values (Figure 1A), which is consistent with the timing of hydraulic conductivity reestablishment in other temperate tree species (Sperry *et al.*, 1988; Miller-Rushing and Primack, 2008). The air injection and dye perfusion experiments also indicate that the VWC change in *B. papyrifera* trunks was related to xylem cavitation. The gradual increase of VWC after air injection in the early spring and a lack of increase in VWC after air injection when leaves were fully expanded further indicate that root pressure plays a critical role in the refilling of winter-embolized vessels in this species. Refilling of winter-embolized vessels requires significant positive pressure because these vessels are filled with CO₂ and O₂ enriched air (in cavitated vessels water vapor is rapidly replaced by air), which is not easy to dissolve (Sperry *et al.*, 1987). It has been shown that in early spring when root pressure is prevented from reaching the crown, by overlapping saw cuts, hydraulic conductivity of *Betula* species cannot recover from winter levels (Sperry, 1993). A significant negative correlation between percentage loss

of hydraulic conductivity and wood water content has been found in *Betula* species (Strati *et al.*, 2003), which is consistent with the winter embolism refilling in *B. papyrifera* trunks as measured by VWC increase using FDR sensors in the present study.

Unlike in vines, no evidence for gas expulsion through xylem “leaks” has been found in trees (Sperry *et al.*, 1988; Hacke and Sauter, 1996). It seems that the dissolution of gas into xylem sap under positive pressure and the diffusion of gas to the outside surface of the branch are the major mechanisms for winter embolism repair in trees (Yang and Tyree, 1992). The results of this study showed that during the active refilling process in early spring, VWC showed large diurnal fluctuations (Appendix Figure B.3A), which might be due to the shrinkage and expansion of air bubbles in the xylem in response to root pressure. The diurnal fluctuation in VWC diminished over a period of two weeks, which was likely due to the slow reduction in bubble size as root pressure forced gas into solution.

Trunk xylem vulnerability

The trunk air injection method is based on the same principles as the conventional air injection method (double-ended pressure sleeve) for determining hydraulic vulnerability in small branches, which measures decrease in hydraulic conductivity of hydrated stems or roots as a function of air pressure surrounding the xylem (Sperry and Saliendra, 1994). The conventional air injection method estimates the pressure needed to induce cavitation by assuming that positive pressure will push air across the pit membranes when it is equal and opposite to the tension that would pull air across the pits in functioning xylem. This idea, which stems from the “air seeding” hypothesis (Zimmermann, 1983), is strongly supported by the close correspondence between vulnerability curves obtained by air

injection and those derived by dehydration methods (Sperry *et al.*, 1996). Similar to branches measured with a double-ended pressure sleeve, the air injection of the trunk resulted in sigmoid curves when wood VWC was plotted against the air pressure (Figure 5.4A).

During the air injection treatment, the high-pressure gas propagates both radially and axially and the pressure builds up in the xylem around the injection point, which was larger than the volume of wood that the FDR moisture probe senses as evidenced by the dye perfusion results (Figure 5.4B). The results suggest that the decrease of VWC in *B. papyrifera* during air injection was mainly due to water being forced out of the vessels other than the surrounding tissue, which is dominantly fiber matrix (Appendix Figure B.6). Although the fiber lumens can contain a considerable amount of water, they may not be well connected to the vessels hydraulically, which otherwise would allow the stored water easily be pulled away by the transpiration stream undermining their water storage function (Holbrook, 1995). Under a pressure higher than that needed for “air-seeding”, water contained in the affected vessels can easily be pushed axially to the neighboring vessels up or down stream of the injection point through pits, while the much shorter length of the fiber cells relative to the vessels may have resulted in the minor contribution of water loss in fiber cells during injection. If the discharge of fiber capillary storage had contributed to the trunk VWC decrease during air injection, a large VWC drop would have been observed at a low pressure range between 0 and 0.6 MPa, which is the theoretical functioning range for fiber water storage (Tyree and Yang, 1990). Together with the dye perfusion results and the agreement between the observed maximum VWC drop during air injection (on average $0.11 \text{ m}^3 \text{ m}^{-3}$ for the March

injection) and the calculated volume fraction of vessel lumen inside the sapwood (15%), it is reasonable to conclude that the measured VWC change during air injection was mainly due to embolism formation in the xylem. The resulting curves from this method can therefore be used as a surrogate for trunk xylem hydraulic “vulnerability curves”.

Correspondence in P_{50} (the pressure at which there is a 50% loss of conductance or reduction in relative VWC change) between small branch hydraulic vulnerability curves and the trunk air injection curves suggests that trunk and branch xylem are equally vulnerable to drought-induced cavitation (Figure 5.4A), although the vessel diameter in trunks is much greater than that of small branches. This suggests, at least for *B. papyrifera*, the absence of “vulnerability segmentation” between the tree trunk and terminal branches. Contradicting results have been found in different species in comparing hydraulic vulnerability of branches of different diameters (Cochard, 1992; Tyree *et al.*, 1993; Sperry and Saliendra, 1994; Sperry and Ikeda, 1997; Choat *et al.*, 2005), which indicates that “vulnerability segmentation” is not a general phenomenon. The ambiguous relationship between conduit size and vulnerability to drought-induced cavitation is consistent with the lack of a necessary causal link between them. Rather, cavitation appears to be structurally correlated with inter-conduit pit membranes that determine the critical pressure or tension for air seedling (Sperry *et al.*, 1996).

The lack of apparent “vulnerability segmentation” however does not necessarily imply that the trunk and peripheral xylem are equally susceptible to drought-induced cavitation in these trees. It has been found that a larger fraction of whole xylem hydraulic resistance resides in the branches than in the trunk (Edwards *et al.*, 1986; Tyree and Sperry, 1988; Tyree, 1988; Tyree *et al.*, 1991; Yang and Tyree, 1994), which results in much steeper

water potential gradients in the small terminal branches than in big stems or the trunk of a transpiring tree. Higher water potential in big stems or the trunk itself is likely to confer a lower risk of cavitation compared to that in small terminal branches. It has also been found that leaves are more vulnerable to drought-induced hydraulic failure than stems (Hao *et al.*, 2008), which suggest that “vulnerability segmentation” may mainly occur between terminal branches and leaves rather than between stems of different sizes (Choat *et al.*, 2005).

Xylem water storage and plant water balance

It has long been known that trees can store water in their xylem tissues with a seasonal and diurnal rhythm of storage and depletion (Landsberg *et al.*, 1976; Roberts, 1976; Waring *et al.*, 1979). Although sap flow and stem dendrometer techniques have been used to estimate the contribution of trunk water storage to daily transpiration (e.g. Goldstein *et al.*, 1998; Scholz *et al.*, 2008), no previous study has provided such a high temporal resolution and continuous measurements nondestructively in functioning trees over a relatively long period. By using the FDR moisture sensor, critical methodological problems inherent in measuring diurnal and seasonal changes of wood water content by sampling with increment coring equipment were avoided (Waring and Running, 1978; Waring *et al.*, 1979). Whenever wood fibers and conduits are cut open, the water contained in them is held only by the capillary forces generated by the radius of curvature of the air-water interfaces of the severed cells and thus can be pulled away by tension in the neighboring functional xylem. The FDR probe installation used in the present study does only minor damage to the xylem, relative to a much larger volume of untouched wood tissues that the probe senses (volume of influence is 0.3 L), and thus provides a

more accurate measurement of diurnal and seasonal changes of xylem tissue water content.

The diurnal fluctuations in trunk VWC indicate the importance of internal water storage to daily water balance in *B. papyrifera* trees. Withdrawal of water from internal storage compartments can account for 10% to 50% daily water use in trees depending on species, ecosystem type and tree size (Goldstein *et al.*, 1998; Meinzer *et al.*, 2004; Scholz *et al.*, 2007; Scholz *et al.*, 2011). The use of internal stored water close to the site of transpiration reduces the apparent hydraulic resistance along the plant water transport pathway and can therefore buffer temporal changes in leaf water status, which in turn can reduce the extent of stomata limitation to photosynthesis (Meinzer, 2002). Water storage as a homeostatic mechanism may be especially important in tall trees as hydraulic resistance increases with tree height (Goldstein *et al.*, 1998; Phillips *et al.*, 2003). Furthermore, daily water withdrawal from storage at times of peak transpiration with later recharge, allows roots to take up water at intermediate rates over a longer period, allowing plants to meet their water needs with less roots than would otherwise be necessary (Tyree and Yang, 1990).

The VWC changed over longer time periods, such as recharge after significant rainfalls and gradual decline thereafter as well as the dramatic decrease that occurred during a summer heat wave (Figures 5.2A and 5.2B), indicates the importance of water storage to plant water balance in longer terms. More interestingly, the water content of the trunks had an overall gradual decrease from leaf flush through the end of the growing season, which may largely be due to changes in fiber capillary water storage since parenchyma ray cells only occupy a very small portion of the xylem cross-section in *B. papyrifera*.

The intercellular spaces inside fibers, fiber tracheids, or tracheids can provide considerable water storage capacitance in trees in the form of capillary storage (Zimmermann, 1983; Tyree and Yang, 1990). Partially air-filled fibers in the xylem of hardwood trees are ideal for water storage, because their slender tips can hold a considerable amount of water. Because capillary tension is inversely related to diameter, the volume of water trapped inside fibers is negatively related to xylem tension; as the tension increases the water menisci of bubbles inside the fiber lumens are pulled toward the narrower tips where larger capillary forces reestablish balance with tension. When water is available and xylem tension decreases the capillary tension in the fiber causes air bubbles to shrink and more water can be stored in the fibers.

Water storage by capillarity only provides an appreciable amount of water over a relatively narrow range of water potentials between 0 and -0.6 MPa (Tyree and Yang, 1990). Water potential measured on *Betula occidentalis* grown in Utah using stem psychrometers showed that midday trunk water potential in August was about -0.6 MPa (Sperry and Pockman, 1993). In the more humid environment of the current study site, one can safely predict that the *B. papyrifera* trunk water potential rarely departs from within this range of 0 to -0.6 MPa, which is not low enough to induce significant wood water loss due to vessel cavitation according the vulnerability curves (Figure 4A) but matches the water potential range in which capillary water storage functions. The slower VWC increase under root pressure in the autumn relative to that happened in the spring also suggest that the seasonal water content decrease in the trunk xylem was not due to cavitation but likely due to the depletion of stored water in fibers, which might be more difficult to refill.

In conclusion, FDR moisture sensors have the potential to detect important processes related to the short-and long-term dynamics of plant water balance in tree trunks. The trunk air injection technique developed based on the FDR measurement of water content seems to be a reliable technique for estimating xylem vulnerability in tree trunks or large stems. Trees subjected to air injection in the early spring in the presence of root pressure partly recovered VWC implying vessel refilling, while those trees air injected in the absence of root pressure showed no recovery of VWC. The contrasting post air injection responses of xylem VWC in trees with and without root pressure indicate the essential role of root pressure in embolism refilling. Comparison of the “vulnerability curves” derived from the trunk air injection and conventional branch hydraulic vulnerability curves found no evidence of “vulnerability segmentation” between the trunk and small branches in *B. papyrifera*.

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Bibliography

- Alder N. N., Pockman W. T., Sperry J. S. & Nuismer S. (1997) Use of centrifugal force in the study of xylem cavitation. *Journal of Experimental Botany* **48**, 665-674.
- Améglio T., Bodet C., Lacoïnte A., Cochard H. (2002) Winter embolism, mechanisms of xylem hydraulic conductivity recovery and springtime growth patterns in walnut and peach trees. *Tree Physiology* **22**, 1211-1220.
- Boyer J. S. (1968) Relationship of water potential to growth of leaves. *Plant Physiology* **43**, 1056-1062.
- Boyer J. S. (1971) Resistance to Water Transport in Soybean, Bean, and Sunflower. *Crop Science* **11**, 403-407.
- Brodersen C. R., McElrone A. J., Choat B., Matthews M. A. & Shackel K. A. (2010) The dynamics of embolism repair in xylem: in vivo visualizations using high-resolution computed tomography. *Plant Physiology* **154**, 1088-1095
- Brodribb T. J. & Holbrook N. M. (2003) Stomatal Closure during Leaf Dehydration, Correlation with Other Leaf Physiological Traits. *Plant Physiology* **132**, 2166-2173
- Brown, H. R. (2013) The theory of the rise of sap in trees: some historical and conceptual remarks. *Physics in Perspective* **15**, 320-358.
- Bucci S. J., Scholz F. G., Goldstein G., Meinzer F. C. & Sternberg L. D. S. L. (2003) Dynamic changes in hydraulic conductivity in petioles of two savanna tree species: factors and mechanisms contributing to the refilling of embolized vessels. *Plant, Cell and Environment* **26**, 1633-1645
- Choat B., Lahr E. C., Melcher P. J., Zwieniecki M. A. & Holbrook N. M. (2005) The spatial pattern of air seeding thresholds in mature sugar maple trees. *Plant, Cell and Environment* **28**, 1082-1089.
- Choat B., Drayton W. M., Brodersen C., Matthews M. A., Shackel K. A., Wada H. & McElrone A. J. (2010) Measurement of vulnerability to water stress-induced cavitation in grapevine: a comparison of four techniques applied to a long-vesseled species. *Plant, Cell and Environment* **33**, 1502-1512.
- Choat B., Jansen S., Brodribb T. J., *et al.* (2012) Global convergence in the vulnerability of forests to drought. *Nature* **491**, 4-8.

- Christman M. A., Sperry J. S. & Adler F. R. (2009) Testing the 'rare pit' hypothesis for xylem cavitation resistance in three species of *Acer*. *New Phytologist* **182**, 664-674.
- Christman M. A., Sperry J. S. & Smith D. D. (2012) Rare pits, large vessels and extreme vulnerability to cavitation in a ring-porous tree species. *New Phytologist* **193**, 713-720.
- Clearwater M. J. & Goldstein G. (2005) Embolism Repair and Long Distance Water Transport. In *Vascular Transport in Plants*. 375-400
- Cobb A. R., Choat B. & Holbrook N. M. (2007) Dynamics of freeze-thaw embolism in *Smilax rotundifolia* (smilacaceae). *American Journal of Botany* **94**, 640-649.
- Cochard H. & Tyree M. T. (1990) Xylem dysfunction in *Quercus*: vessel sizes, tyloses, cavitation and seasonal changes in embolism. *Tree Physiology* **6**, 393-407.
- Cochard H. (1992) Vulnerability of several conifers to air embolism. *Tree Physiology* **11**, 73-83.
- Cochard H., Cruiziat P. & Tyree, M. T. (1992) Use of positive pressures to establish vulnerability curves. Further support for the air-seeding hypothesis and implications for pressure-volume analysis. *Plant Physiology* **100**, 205-209.
- Cochard H., Damour G., Bodet C., Tharwat I., Poirier M. & Améglio T. (2005) Evaluation of a new centrifuge technique for rapid generation of xylem vulnerability curves. *Physiologia Plantarum* **124**, 410-418.
- Cochard H., Herbette S., Barigah T., Badel E., Ennajeh M. & Vilagrosa A. (2010) Does sample length influence the shape of xylem embolism vulnerability curves? A test with the Cavitron spinning technique. *Plant, Cell & Environment* **33**, 1543-1552.
- Cox R. M. & Malcolm J. W. (1997) Effects of duration of a simulated winter thaw on dieback and xylem conductivity of *Betula papyrifera*. *Tree physiology* **17**, 389-396.
- Crombie D. S., Hipkins M. F. & Milburn, J. A. (1985) Gas penetration of pit membranes in the xylem of *Rhododendron* as the cause of acoustically detectable sap cavitation. *Functional Plant Biology* **12**, 445-453.
- Cruiziat P., Cochard H. & Améglio T. (2002) Hydraulic architecture of trees: main concepts and results. *Annals of Forest Science* **59**, 723-752

- Czarnomski N. M, Moore G. W, Pypker T. G, Licata J. & Bond B. J. (2005) Precision and accuracy of three alternative instruments for measuring soil water content in two forest soils of the Pacific Northwest. *Canadian Journal of Forest Research* **35**, 1867-1876.
- Dixon H. H. (1914) *Transpiration and the Ascent of Sap in Plants*. Macmillan and co., London
- Domec J. C. & Gartner B. L. (2001) Cavitation and water storage capacity in bole xylem segments of mature and young Douglas-fir trees. *Trees* **15**, 204-214.
- Edwards W. R. N., Jarvis P. G., Landsberg J. J. & Talbot H. (1986) A dynamic model for studying flow of water in single trees. *Tree Physiology* **1**, 309-324.
- Ennajeh M., Simões F., Khemira H. & Cochard, H. (2011) How reliable is the double-ended pressure sleeve technique for assessing xylem vulnerability to cavitation in woody angiosperms?. *Physiologia Plantarum* **142**, 205-210.
- Esau K. (1953) *Plant Anatomy*, John Wiley & Sons, Inc. New York, NY, USA.
- Ewers F. W. (1985) Xylem structure and water conduction in conifer trees, dicot trees, and lianas. *IAWA Bulletin* **6**, 309-317.
- Ewers F. W., Améglio T., Cochard H., Beaujard F., Martignac M., Vandame M., Bodet C. & Cruiziat P. (2001) Seasonal variation in xylem pressure of walnut trees: root and stem pressures. *Tree physiology* **21**, 1123-1132.
- Goldstein G., Andrade J. L., Meinzer F. C., Holbrook N. M., Cavelier J., Jackson P. & Celis A. (1998) Stem water storage and diurnal patterns of water use in tropical forest canopy trees. *Plant, Cell & Environment* **21**, 397-406.
- Greenidge K. N. H. (1952) An approach to the study of vessel length in hardwood species. *American Journal of Botany* **39**, 570-574.
- Hacke U. & Sauter J. J. (1996) Xylem dysfunction during winter and recovery of hydraulic conductivity in diffuse-porous and ring-porous trees. *Oecologia* **105**, 435-439.
- Hacke U. G. & Sperry J. S. (2003) Limits to xylem refilling under negative pressure in *Laurus nobilis* and *Acer negundo*. *Plant, Cell & Environment* **26**, 303-311.
- Hacke U. G., Sperry J. S., Wheeler J. K. & Castro L. (2006) Scaling of angiosperm xylem structure with safety and efficiency. *Tree Physiology* **26**, 689-701.

- Hammel H. T. (1967) Freezing of xylem sap without cavitation. *Plant Physiology* **42**, 55-66.
- Hao G., Hoffmann W. A., Scholz F. G., Bucci S. J., Meinzer F. C., Franco A.C., Cao K. & Goldstein G. (2008) Stem and leaf hydraulics of congeneric tree species from adjacent tropical savanna and forest ecosystems. *Oecologia* **155**, 405-415.
- Hao G., Wheeler J. K., Holbrook N. M. & Goldstein G. (2013) Investigating xylem embolism formation, refilling and water storage in tree trunks using frequency domain reflectometry. *Journal of experimental botany* **64**, 2321-2332.
- Holbrook N. M. (1995) Stem water storage. In: Gartner BL, ed. *Plant stems: physiology and functional morphology*. San Diego: Academic press, 151-174.
- Holbrook N. M. & Zwieniecki M. A. (1999) Embolism repair and xylem tension: Do We need a miracle? *Plant Physiology* **120**, 7-10
- Holbrook N. M., Ahrens E. T., Burns M. J. & Zwieniecki M. A. (2001) In vivo observation of cavitation and embolism repair using magnetic resonance imaging. *Plant Physiology* **126**, 27-31.
- Jacobsen A. L. & Pratt R. B. (2012) No evidence for an open vessel effect in centrifuge-based vulnerability curves of a long-vesseled liana (*Vitis vinifera*). *New Phytologist* **194**, 982-990.
- James S. A., Meinzer F. C., Goldstein G., Woodruff D., Jones T., Restom T., Mejia M., Clearwater M. & Campanello P. (2003) Axial and radial water transport and internal water storage in tropical forest canopy trees. *Oecologia* **134**, 37-45.
- Jaquish L. L. & Ewers F. W. (2001) Seasonal conductivity and embolism in the roots and stems of two clonal ring-porous trees, *Sassafras albidum* (Lauraceae) and *Rhus typhina* (Anacardiaceae). *American Journal of Botany* **88**, 206-212.
- Jensen K. H., Huggett B. A. & Holbrook N. M. (2013) Xylem vessels: length distribution and air seeding pressure. *unpublished*
- Johnson D. M., Woodruff D. R., McCulloh K. A. & Meinzer F. C. (2009) Leaf hydraulic conductance, measured in situ, declines and recovers daily: leaf hydraulics, water potential and stomatal conductance in four temperate and three tropical tree species. *Tree Physiology* **29**, 879-887.

- Kizito F., Campbell C. S., Campbell G. S., Cobos D. R., Teare B. L., Carter B. & Hopmans J.W. (2008) Frequency, electrical conductivity and temperature analysis of a low-cost capacitance soil moisture sensor. *Journal of Hydrology* **352**, 367-378.
- Kramer P. J. & Boyer J. S. (1995) *Water Relations of Plants and Soils*, Academic Press San Diego, CA, USA.
- Landsberg J. J., Blanchard T. W., Warritt B. (1976) Studies on movement of water through apple trees. *Journal of Experimental Botany* **27**, 579-596.
- Lens F., Sperry J. S., Christman M. A., Choat B., Rabaey D. & Jansen S. (2011) Testing hypotheses that link wood anatomy to cavitation resistance and hydraulic conductivity in the genus *Acer*. *New Phytologist* **190**, 709-723.
- Li Y. Y., Shi H. & Shao M. A. (2010) Cavitation resistance of dominant trees and shrubs in Loess hilly region and their relationship with xylem structure. *Journal of Beijing Forestry University* **32**, 8-13.
- Lopez O. R., Kursar T. A., Cochard H. & Tyree M. T. (2005) Interspecific variation in xylem vulnerability to cavitation among. *Tree Physiology* **25**: 1553-1562
- Magnani F., Borghetti M. (1995) Interpretation of seasonal changes of xylem embolism and plant hydraulic resistance in *Fagus sylvatica*. *Plant, Cell and Environment* **18**, 689-696.
- Maherali H., Pockman W. T. & Jackson R. B. (2004) Adaptive variation in the vulnerability of woody plants to xylem cavitation. *Ecology* **85**, 2184-2199.
- McElrone A. J., Brodersen C. R., Alsina M. M., Drayton W. M., Matthews M. A., Shackel K. A., Wada H., Zufferey V. & Choat B. (2012) Centrifuge technique consistently overestimates vulnerability to water stress-induced cavitation in grapevines as confirmed with high-resolution computed tomography. *New Phytologist* **196**, 661-665.
- Meinzer F. C. (2002) Co-ordination of vapour and liquid phase water transport properties in plants. *Plant, Cell and Environment* **25**, 265-274.
- Meinzer F. C., Clearwater M. J. & Goldstein G. (2001) Water transport in trees: current perspectives, new insights and some controversies. *Environmental and Experimental Botany* **45**, 239-262.
- Meinzer F. C., James S. A., Goldstein G. (2004) Dynamics of transpiration, sap flow and use of stored water in tropical forest canopy trees. *Tree Physiology* **24**, 901-909.

- Melcher P. J., Zwieniecki M. A. & Holbrook N. M. (2003) Vulnerability of Xylem Vessels to Cavitation in Sugar Maple . Scaling from Individual Vessels to Whole Branches. *Plant Physiology* **131**, 1775-1780.
- Miller-Rushing A. J. & Primack R. B. (2008) Effects of winter temperatures on two birch (*Betula*) species. *Tree Physiology* **28**, 659-664.
- Nardini A., Lo Gullo M. A. & Salleo S. (2011) Refilling embolized xylem conduits: is it a matter of phloem unloading?. *Plant Science* **180**, 604-611.
- Oertli J. J. (1971) The stability of water under tension in the xylem. *Zeitschrift für Pflanzenphysiologie* **65**, 195-209.
- Phillips N. G., Ryan M. G., Bond B. J., McDowell N. G., Hinckley T. M. & Cermák J. (2003) Reliance on stored water increases with tree size in three species in the Pacific Northwest. *Tree Physiology* **23**, 237-245.
- Pockman W. T., Sperry J. S. & O'Leary J. W. (1995) Sustained and significant negative water pressure in xylem. *Nature* **378**, 715-716.
- Pockman W. T. & Sperry J. S. (2000) Vulnerability to xylem cavitation and the distribution of Sonoran desert vegetation. *American Journal of Botany* **87**, 1287-1299.
- Roberts J. (1976) Examination of the quantity of water stored in mature *Pinus sylvestris* L. trees. *Journal of Experimental Botany* **27**, 473-479.
- Rockwell F. E. (2010). *Leaf Water Transport* (Doctoral dissertation, Harvard University).
- Salleo S., Lo Gullo M., De Paoli D. & Zippo M. (1996) Xylem recovery from cavitation-induced embolism in young plants of *Laurus nobilis*: a possible mechanism. *New Phytologist* **132**, 47-56.
- Scholz F. G., Bucci S. J., Goldstein G., Meinzer F. C., Franco A. C. & Miralles-Wilhelm F. (2007) Biophysical properties and functional significance of stem water storage tissue in neo-tropical savanna trees. *Plant, Cell and Environment* **30**, 236-248.

- Scholz F. G., Bucci S. J., Goldstein G., Meinzer F. C., Franco A. C., Miralles-Wilhelm F. (2008) Temporal dynamics of stem expansion and contraction in savanna trees: withdrawal and recharge of stored water. *Tree Physiology* **28**, 469-480.
- Scholz F. G., Phillips N. G., Bucci S. J., Meinzer F. G. & Goldstein G. (2011) Hydraulic capacitance: biophysics and functional significance of internal water sources in relation to tree size. In: Meinzer FC, Lachenbruch B, Dawson TE, eds. *Size- and age-related changes in tree structure and function*. New York: Springer, 341-362.
- Schulze E. D., Čermák J., Matyssek M., Penka M., Zimmermann R., Vasicek F., Gries W. & Kučera J. (1985) Canopy transpiration and water fluxes in the xylem of the trunk of *Larix* and *Picea* trees—a comparison of xylem flow, porometer and cuvette measurements. *Oecologia* **66**, 475-483.
- Scoffoni C., McKown A. D., Rawls M. & Sack L. (2012) Dynamics of leaf hydraulic conductance with water status: quantification and analysis of species differences under steady state. *Journal of Experimental Botany* **63**, 643-658
- Secchi F. & Zwieniecki M. A. (2010) Patterns of PIP gene expression in *Populus trichocarpa* during recovery from xylem embolism suggest a major role for the PIP1 aquaporin subfamily as moderators of refilling process. *Plant, Cell & Environment* **33**, 1285-1297.
- Secchi F. & Zwieniecki M. A. (2011) Sensing embolism in xylem vessels: the role of sucrose as a trigger for refilling. *Plant, Cell and Environment* **34**, 514-524
- Sokal R. R. & Rohlf F. J. (1995) *Biometry*, 3rd ed. W.H. Freeman and Company, New York, NY.
- Sperry J. S., Donnelly J. R. & Tyree M. T. (1988) A method for measuring hydraulic conductivity and embolism in xylem. *Plant, Cell and Environment* **11**, 35-40.
- Sperry J. S., Holbrook N. M. & Zimmermann M. H., Tyree M. T. (1987) Spring filling of xylem vessels in wild grapevine. *Plant Physiology* **83**: 414-417
- Sperry J. S. & Sullivan J. E. M. (1992) Xylem embolism in response to freeze-thaw cycles and water stress in ring-porous, diffuse-porous, and conifer species. *Plant Physiology* **100**, 605-613.
- Sperry J. S. & Pockman W. T. (1993) Limitation of transpiration by hydraulic conductance and xylem cavitation in *Betula occidentalis*. *Plant, Cell and Environment* **16**, 279-287.

- Sperry J. S. (1993) Winter xylem embolism and spring recovery in *Betula cordifolia*, *Fagus grandifolia*, *Abies balsamifera*, and *Picea rubens*. In: Raschi A, Borghetti M, Grace J, eds. *Water transport in plants under climatic stress*. Cambridge: Cambridge University Press, 86-98.
- Sperry J. S., Nichols K. L., Sullivan J. E. & Eastlack S. E. (1994) Xylem embolism in ring-porous, diffuse-porous, and coniferous trees of northern Utah and interior Alaska. *Ecology* **75**: 1736-1752
- Sperry J. S. & Saliendra N. Z. (1994) Intra- and inter-plant variation in xylem cavitation in *Betula occidentalis*. *Plant, Cell and Environment* **17**, 1233-1241.
- Sperry J. S., Saliendra N. Z., Pockman W. T., Cochard H., Cruiziat P., Davis S. D., Ewers F.W. & Tyree M. T. (1996) New evidence for large negative xylem pressures and their measurement by the pressure chamber method. *Plant, Cell and Environment* **19**, 427-436.
- Sperry J. S. & Ikeda T. (1997) Xylem cavitation in roots and stems of Douglas-fir and white fir. *Tree Physiology* **17**, 275-280.
- Sperry J. S. (2000) Hydraulic constraints on plant gas exchange. *Agricultural and Forest Meteorology* **104**, 13-23.
- Sperry J. S. & Hacke U. G. (2004) Analysis of circular bordered pit function I. Angiosperm vessels with homogenous pit membranes. *American Journal of Botany* **91**, 369-385.
- Sperry J. S., Christman M. A., Torres-Ruiz J. M., Taneda H. & Smith D. D. (2012) Vulnerability curves by centrifugation: is there an open vessel artefact, and are 'r' shaped curves necessarily invalid?. *Plant, Cell & Environment* **35**, 601-610.
- Strati S., Patiño S., Slidders C., Cundall E. P. & Mencuccini M. (2003) Development and recovery from winter embolism in silver birch: seasonal patterns and relationships with the phenological cycle in oceanic Scotland. *Tree Physiology* **23**, 663-673.
- Taneda H. & Sperry J. S. (2008) A case-study of water transport in co-occurring ring- versus diffuse-porous trees: contrasts in water-status, conducting capacity, cavitation and vessel refilling. *Tree Physiology* **28**, 1641-1651.

- Tobin M. F., Pratt R. B., Jacobsen A. L. & De Guzman M. E. (2012) Xylem vulnerability to cavitation can be accurately characterised in species with long vessels using a centrifuge method. *Plant Biology* **15**, 496-504.
- Torres-Ruiz J. M., Sperry J. S. & Fernández J. E. (2012) Improving xylem hydraulic conductivity measurements by correcting the error caused by passive water uptake. *Physiologia plantarum* **146**, 129-135
- Tyree M. T. (1988) A dynamic model for water flow in a single tree: evidence that models must account for hydraulic architecture. *Tree Physiology* **4**, 195-217.
- Tyree M. T. & Sperry J. S. (1988) Do woody plants operate near the point of catastrophic xylem dysfunction caused by dynamic water stress? Answers from a model. *Plant physiology* **88**, 574-580.
- Tyree M. T. & Sperry J. S. (1989) Vulnerability of xylem to cavitation and embolism. *Annual review of plant biology* **40**, 19-36.
- Tyree M. T. & Yang S. D. (1990) Water-storage capacity of *Thuja*, *Tsuga* and *Acer* stems measured by dehydration isotherms. The contribution of capillary water and cavitation. *Planta* **182**, 420-426.
- Tyree M. T., Ewers F. W. (1991) The hydraulic architecture of trees and other woody plants. *New Phytologist* **119**, 345-360.
- Tyree M. T., Snyderman D. A., Wilmot T. R. & Machado J-L. (1991) Water relations and hydraulic architecture of a tropical tree (*Schefflera morototoni*). Data, models, and a comparison with two temperate species (*Acer saccharum* and *Thuja occidentalis*). *Plant Physiology* **96**, 1105-1113.
- Tyree M. T., Cochard H., Cruiziat P., Sinclair B. & Améglio T. (1993) Drought-induced leaf shedding in walnut: evidence for vulnerability segmentation. *Plant, Cell and Environment* **16**, 879-882.
- Tyree M. T., Davis S. & Cochard, H. (1994) Biophysical perspectives of xylem evolution- is there a tradeoff of hydraulic efficiency for vulnerability to dysfunction? *IAWA Journal* **15**, 335-360.
- Tyree M. T., Salleo S., Nardini A., Lo Gullo M. A. & Mosca R. (1999) Refilling of embolized vessels in young stems of laurel. Do We need a new paradigm? *Plant Physiology* **120**, 11-22

- Vogel S. (1988) *Life's devices: the physical world of animals and plants*. Princeton: Princeton University Press.
- Wang J., Ives N. E., Lechowicz M. J. (1992) The relation of foliar phenology to xylem embolism in trees. *Functional Ecology* **6**, 469-475.
- Waring R. H. & Running S. W. (1978) Sapwood water storage: its contribution to transpiration and effect upon water conductance through the stems of old-growth Douglas-fir. *Plant, Cell and Environment* **1**, 131-140.
- Waring R. H., Whitehead D., Jarvis P. G. (1979) The contribution of stored water to transpiration in Scots pine. *Plant, Cell and Environment* **2**, 309-317.
- Wheeler J. K., Sperry J. S., Hacke U. G. & Hoang N. (2005) Inter-vessel pitting and cavitation in woody Rosaceae and other vesselled plants: a basis for a safety versus efficiency trade-off in xylem transport. *Plant, Cell & Environment* **28**, 800-812.
- Wheeler J. K., Huggett B. A., Tofte A. N., Rockwell F. E. & Holbrook N. M. (2013) Cutting xylem under tension or supersaturated with gas can generate PLC and the appearance of rapid recovery from embolism. *Plant, Cell & Environment* **36**, 1938-1949.
- Yang S. D. & Tyree M. T. (1992) A theoretical model of hydraulic conductivity recovery from embolism with comparison to experimental data on *Acer saccharum*. *Plant, Cell and Environment* **15**, 633-643.
- Yang S. D. & Tyree M. T. (1994) Hydraulic architecture of *Acer saccharum* and *A. rubrum*: comparison of branches to whole trees and the contribution of leaves to hydraulic resistance. *Journal of Experimental Botany* **45**, 179-186.
- Zimmermann M. H. (1983) *Xylem Structure and the Ascent of Sap* 1st edn, Springer-Verlag, Berlin, Germany and New York, NY, USA.
- Zufferey V., Cochard H., Ameglio T., Spring J. L. & Viret, O. (2011) Diurnal cycles of embolism formation and repair in petioles of grapevine (*Vitis vinifera* cv. Chasselas). *Journal of Experimental Botany* **62**, 3885-3894.
- Zwieniecki M. A. & Holbrook N. M. (1998) Diurnal variation in xylem hydraulic conductivity in white ash (*Fraxinus americana* L.), red maple (*Acer rubrum* L.) and red spruce (*Picea rubens* Sarg.). *Plant, Cell and Environment* **21**, 1173-1180
- Zwieniecki M. A. & Holbrook N. M. (2009) Confronting Maxwell's demon: biophysics of xylem embolism repair. *Trends in Plant Science* **14**, 530-534

Appendix A

Supplementary materials to Chapter 3; Cutting xylem under tension or supersaturated with gas can generate PLC and the appearance of rapid recovery from embolism.

METHODS DETAILS

Maximum vessel length

Maximum vessel length was measured for each species following Greenidge (1952) Branches, 1-2 meters in length, were cut in air, covered with large plastic bags, and returned to the laboratory. There the tip was cut off and the cut end shaved with a razor blade. This cut end was fitted to tubing and attached to air pressurized to approximately 0.1 MPa. The basal end of the branch was then placed under water and successively shortened by cutting off 1 cm long segments until the first stream of bubbles was detected. The distance between the two cut ends was taken as the maximum vessel length for that segment. Three-to-eight branches per species were measured.

Hydraulic measurements

Samples for flow measurement 4-14 cm in length (one to two internodes long) were cut from sample branches under tap water (HF) or DI water (CC). The basal and apical ends were decorticated and the ends shaved with a fresh razor blade, wrapped in parafilm (to ensure a good seal) and fitted into silicone tubing to attach to the hydraulic apparatus. For stems containing more than one node the petioles were removed under water and the scars sealed with cyanoacrylate glue (Krazyglue all purpose, Elmer's Products Inc.).

Hydraulic conductance measurements were made by measuring the flow driven by a gravity head onto a balance (Sartorius CPA224S at HF, CPA225D at CC) on which the mass change was recorded every second (Sperry, Donnelly & Tyree 1988). To account for stem uptake from the balance, three flow measurements were made; initial and final measurements with no pressure across the segment, and a pressurized flow measurement with a gravity head of 25-40 cm. The mean flow of the initial and final measurements was then deducted from the value of the pressurized flow measurement (Torres-Ruiz, Sperry, & Fernández 2012). This compensation only made a difference in cases where the stems were narrow and dry (most notably in several of the centrifuge measurements at high tension), when the stems were well hydrated, the magnitude of these initial and final measurements was less than 5% of the measurement made with pressure.

The measurement solution was ultra-filtered de-ionized water (HF: Barnstead RO Reverse Osmosis System, series 1266, model D12661; CC: Millipore MilliQ UV plus) with KCl added to make a 20 mM solution, re-filtered through a 0.2 μm filter (Pall acrodisc syringe filters). Maximum conductance for each stem segment was determined by measurement after flushing for 30 minutes at 0.14 MPa, with a filtered 20 mM KCl solution (HF), or for 30 minutes at 0.1 to 0.12 MPa with the same solution degassed (CC).

Xylem pressure measurements

Xylem pressure was measured using a pressure chamber (HF: PMS model 1000; CC: Soil Moisture Equipment Corp.). At HF, to avoid inducing embolism in the measurement segments, the material (small shoots for maple and birch, individual leaves for ash) used to measure xylem pressure was collected from an adjacent limb concurrently with the branches collected for hydraulic measurements. The xylem pressure samples were sealed in plastic bags immediately after removal from the tree and allowed to equilibrate for approximately one hour (Rockwell 2010). For the 2012 diurnal maple experiment and the 2011 crown irrigation of white ash the xylem pressure samples were sealed in foil-covered bags on the tree several hours prior to sampling. For both maple species, pressure chamber measurements were made on leafy side branches (less than 1 cm in length) from which the bark had been removed. This was done because maple petioles frequently produce a foamy exudate when pressurized that makes it difficult to obtain an accurate balancing pressure. In birch and ash individual leaves were measured.

Centrifuge vulnerability curves

The vulnerability to cavitation of red maple and sugar maple branches was measured using the centrifuge method (Alder, Pockman, Sperry, & Nuismer 1997) for comparison to the two types of bench dehydration. Both maples were sampled from the rain-fed populations (CC). In all cases, long branches (> 0.6 m) were cut from trees in air using pole pruners and the branches immediately sealed in plastic bags for transport to the laboratory. In the laboratory the base of the stem was cut several times under water as described above and xylem tensions allowed to relax for 30 minutes before the measurement samples were cut under water. Stem conductance was measured, the stems

were flushed and the conductance was re-measured to obtain a maximum conductance, which was similar to the initial conductance for all the stems measured. The stems were then spun in a custom built rotor (Alder *et al.* 1997) to specific tensions and the conductance re-measured after each spin until the measured conductance was less than 10% of the maximum conductance.

Air injection

For red maple, forked branches > 1 m long were cut from irrigated trees (CC) in the morning (8 AM to 9:30 AM). A short side-shoot, 10 to 20 cm from the base of the branch was removed to determine xylem tension at the time of harvest. The xylem tension was relaxed as in the bench drying curves and a second side-shoot was cut to determine the post-rehydration tension. A one-internode-long sample of current-year growth on each fork (> 40 cm apart) was selected; one for pressurization, and the second for background PLC. The fork containing the background internode was cut under water prior to pressurization. A 5 mm wide band around the central portion of both samples was decorticated to ensure gas penetration (Cochard, Cruiziat, & Tyree 1992; Sperry & Saliendra 1994). The exposed xylem on the background internode was immediately wrapped in parafilm, and a custom-made air injection chamber was assembled around the internode selected for pressurization. All the remaining leaves on both samples were then draped with damp paper towels and the whole branch wrapped in black plastic. The air injection chamber was then pressurized with N₂ gas, at a rate of approximately 0.01 MPa per second; target pressures (i.e., 0.1, 1, or 4MPa) were held for 20 minutes, followed by depressurization at a similar rate.

After complete depressurization of the chamber, the stem was cut under DI water on both sides of the chamber. The decorticated band on the internode was then wrapped in parafilm and the segment floated in a petri dish of DI water for seventy-five minutes prior to measurement of hydraulic conductance (described above). In all cases a rush of bubbles emerged from the cut end upon cutting, and the rate of bubble emergence from the cut end decreased over time.

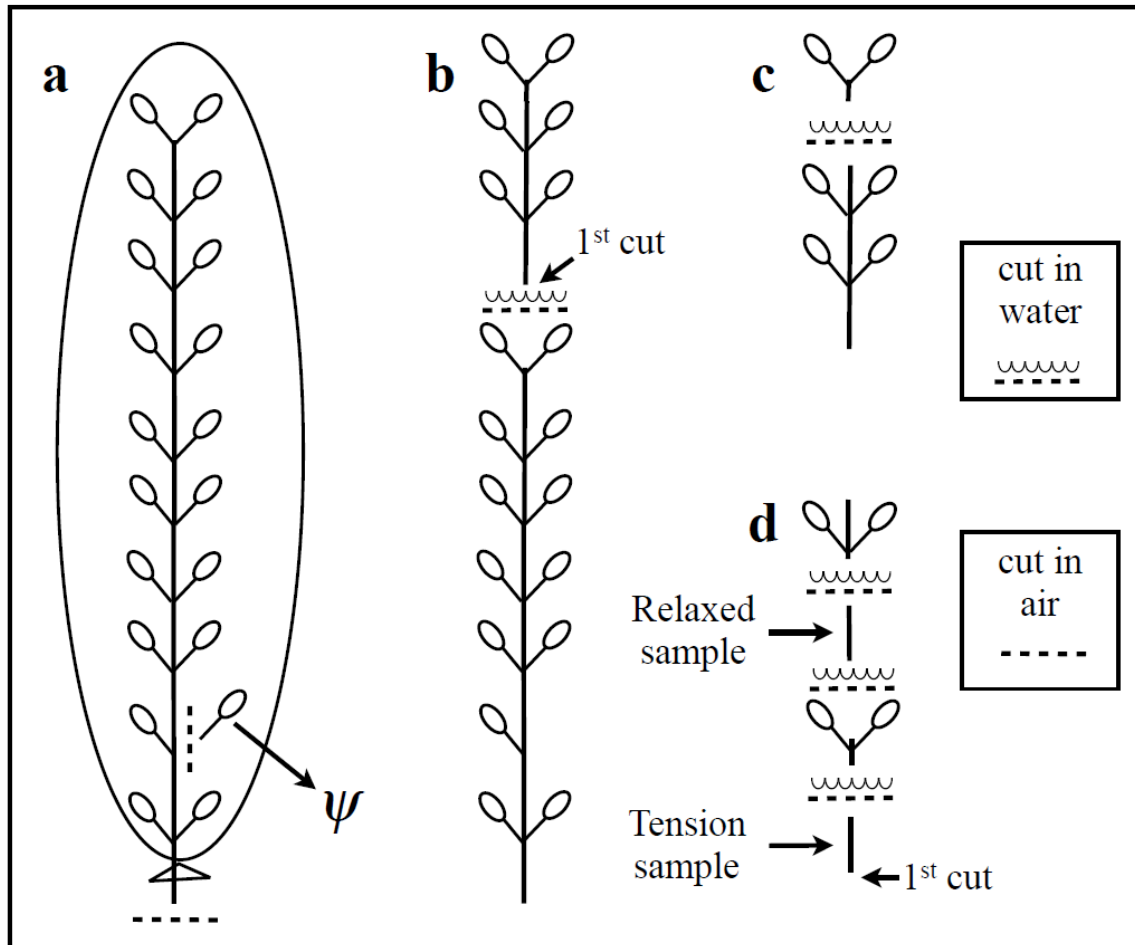
For the delayed cut treatment the branch and chamber were left undisturbed for seventy-five minutes following depressurization of the chamber. After this period, water potential was measured to verify that the branch had not dried out during the delay period. The sample internode that had been pressurized was then harvested from the experimental branch, and the decorticated region wrapped in parafilm. For the delayed cuts, no rush of bubbles on cutting was observed. Average on-tree, relaxed, and post pressurization plus seventy-five minute delay xylem tensions were 0.21MPa, 0.053MPa, and 0.042MPa, demonstrating that the samples did not dry out during the treatments.

For sugar maple, potted trees (averaging 2 m in height) were brought indoors, thoroughly watered and sealed in plastic bags for twelve to sixteen hours. After this hydration period, the aboveground portion of the plant was cut underwater and internodes of current-year growth were selected for background (at >40cm distal to the initial cut) and treatment (at >40cm distal to the background segment) measurement segments. Sample internodes were then processed as described above. Average post-hydration and post-treatment xylem tensions were 0.049MPa and 0.041MPa, respectively.

Statistics

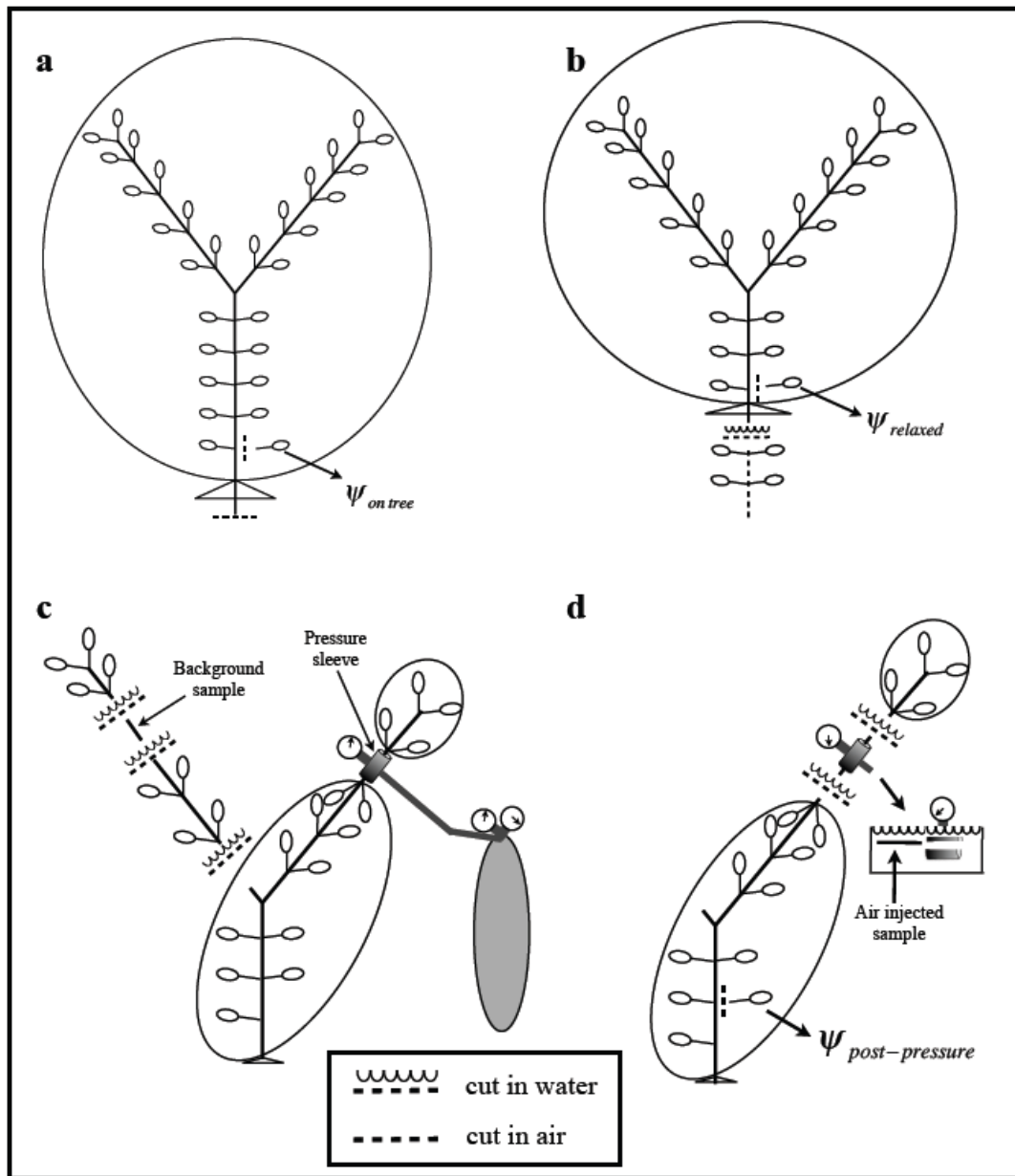
All of the experiments were undertaken as planned comparisons between two treatments (afternoon versus morning, “native tension” versus “relaxed tension”, and immediate cut versus delayed cut). As in each case we expected that the PLC of the former in each treatment pair should exceed the latter, we employed one-sided T-tests, after an arcsine transformation of the percentage data (Sokal & Rohlf 1995). When stems were well hydrated, we frequently encountered small negative PLC values, which we interpret as arising from measurement and flushing error. As we could not discover an unbiased method for handling these negative values, we analyzed all data with both the negative PLC values (calculated as the difference between k_f and k_i normalized by the maximum conductivity) and with negative PLC values set to zero, and finding no difference in our conclusions between these two approaches, applied the standard technique of treating all negative PLC values as zero throughout our final analyses. We expect the error would be important only in attempting to statistically resolve small differences in very low PLCs, which we do not attempt here. All transformations and comparisons were computed in SPSS (IBM SPSS statistics version 20, 2011).

Figure A.1



Rapid relaxation protocol: **a:** a branch dried to the target pressure range was sealed in a plastic bag for thirty minutes to equilibrate and then xylem pressure was measured. **b,c:** The branch was cut under water at both ends of the three internode sample segment, the leaves sliced between the tertiary veins, and the segment relaxed for two minutes. **d:** After the two minute relaxation period the relaxed sample was excised and the PLCs of the tension sample and the relaxed sample were measured.

Figure A.2



Air-injection protocol: **a**: branches removed from a tree are sampled for initial xylem pressure. **b**: The branch base was trimmed under water (20 cm) and the tension relaxed with the branch in a plastic bag for thirty minutes, after which the xylem pressure was measured. **c**: The fork containing the background sample was removed under and covered with plastic bags to ensure that no changes occurred in the samples during the time necessary for air injection. A pressure sleeve was attached to the air-injection fork and pressure was applied for twenty minutes. **d**: Following depressurization, xylem pressure was measured, and the sample segment was excised from the stem and allowed to rest for either two minutes or seventy-five minutes before measuring PLC. Both the background sample and the air-injected sample were measured after the resting period.

Figure A.3

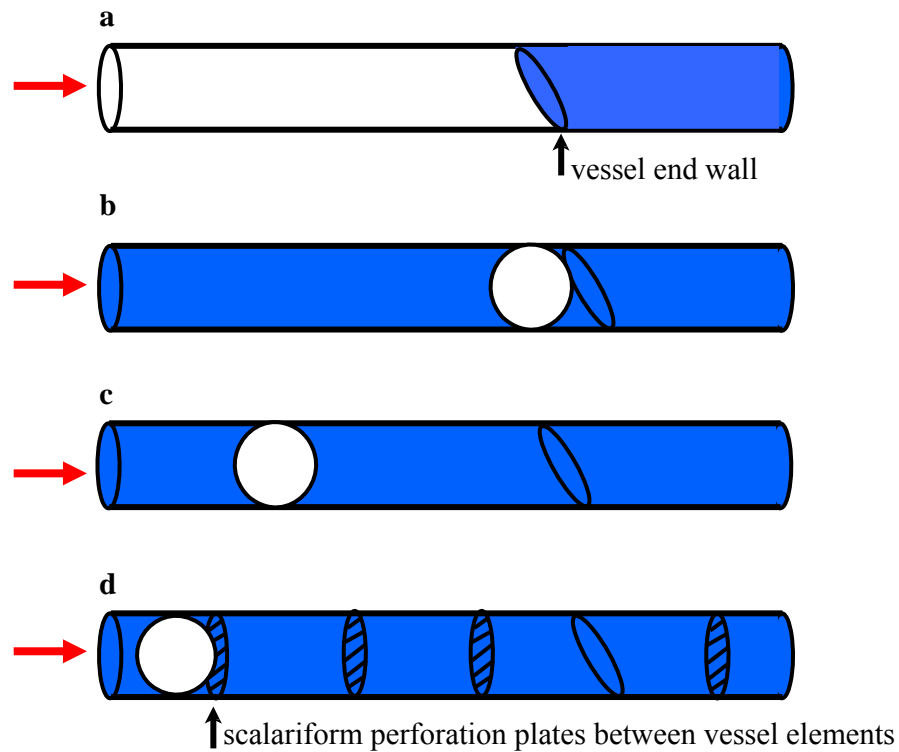


Illustration of the potential outcomes of an air bubble entrained by fluid rushing into the cut surface (red arrows) of a xylem vessel excised under water. **a:** The tension in the xylem fluid is sufficient to cause the bubble to expand and fill the opened vessel to the endwall where intervessel pit membranes prevent the spread of gas throughout the intact xylem. **b:** The tension in the fluid is insufficient to cause the bubble to expand, but it is drawn the length of the vessel and lodges against the endwall. **c:** The bubble is drawn only partly into the opened vessel and may be excised if a sufficient length of the stem is trimmed before measurement. **d:** Scalariform perforation plates (as in paper birch) may prevent bubbles from traveling deep into the opened vessel and thus may be easily removed by shaving the ends before measurement.

Appendix B

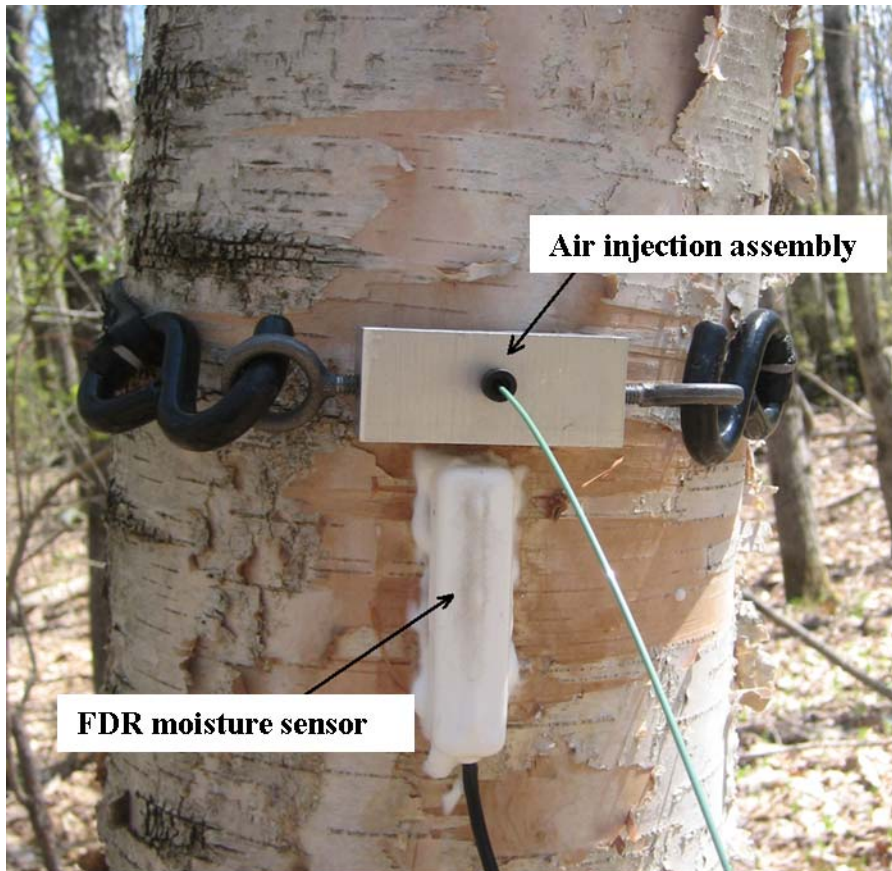
Supplementary materials to Chapter 5; Investigating xylem embolism formation, refilling and water storage in tree trunks using frequency domain reflectometry.

Method for the calibration

Two *B. papyrifera* trees were felled (one in March and one in June 2012) and segments of the trunks were transported to the laboratory while kept under water. A block of sapwood with the above-mentioned dimensions was cut from the segment using an electric saw and three holes for the sensor prongs were drilled using an electric drill. A drill guide described in the article was used to facilitate the drilling. The wood block was then submerged in deionized water under vacuum overnight for hydration. The fresh weight of the block was then measured to the nearest 0.1 g using a Sartorius balance (model 3808 MP8/8-1, Sartorius, Göttingen, Germany) and the volume was determined using water displacement method. The FDR sensor, with weight pre-determined, was then installed in the wood block and the VWC data were recorded using the EM50 data logger. The wood block was then allowed to dry on a lab bench for several hours (depending on the observed weight loss) and then wrapped completely using parafilm and placed in a ziplock bag overnight for equilibration before weight and VWC (by FDR sensor) were measured again. This process was repeated and five sets of weight and

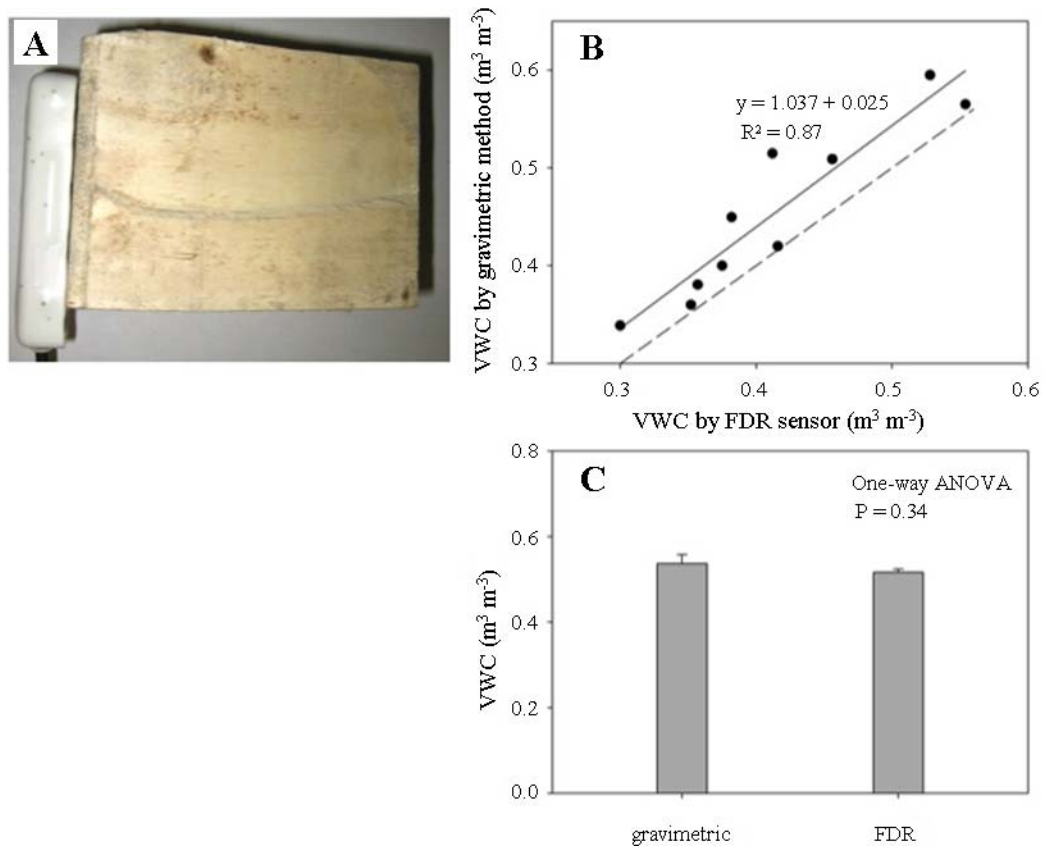
VWC measurements were obtained for each wood block. The wood block was then oven dried at 60 °C for one week before dry weight was measured. The VWC was calculated as (fresh weight - dry weight)/fresh volume. Increment tree cores were taken from five trees (different from the trees used for long-term monitoring) in early spring and VWC was calculated gravimetrically to compare with those measured by the FDR sensors. The deviation (ca. 0.04 m³ m⁻³) between gravimetric and FDR measurements (Figure B.2B) may have resulted from a slightly smaller wood block volume relative to the volume of influence by the FDR sensor. The *in situ* FDR VWC measurements in tree trunks did not differ significantly from that measured gravimetrically on tree cores (Figure B.2C).

Figure B.1



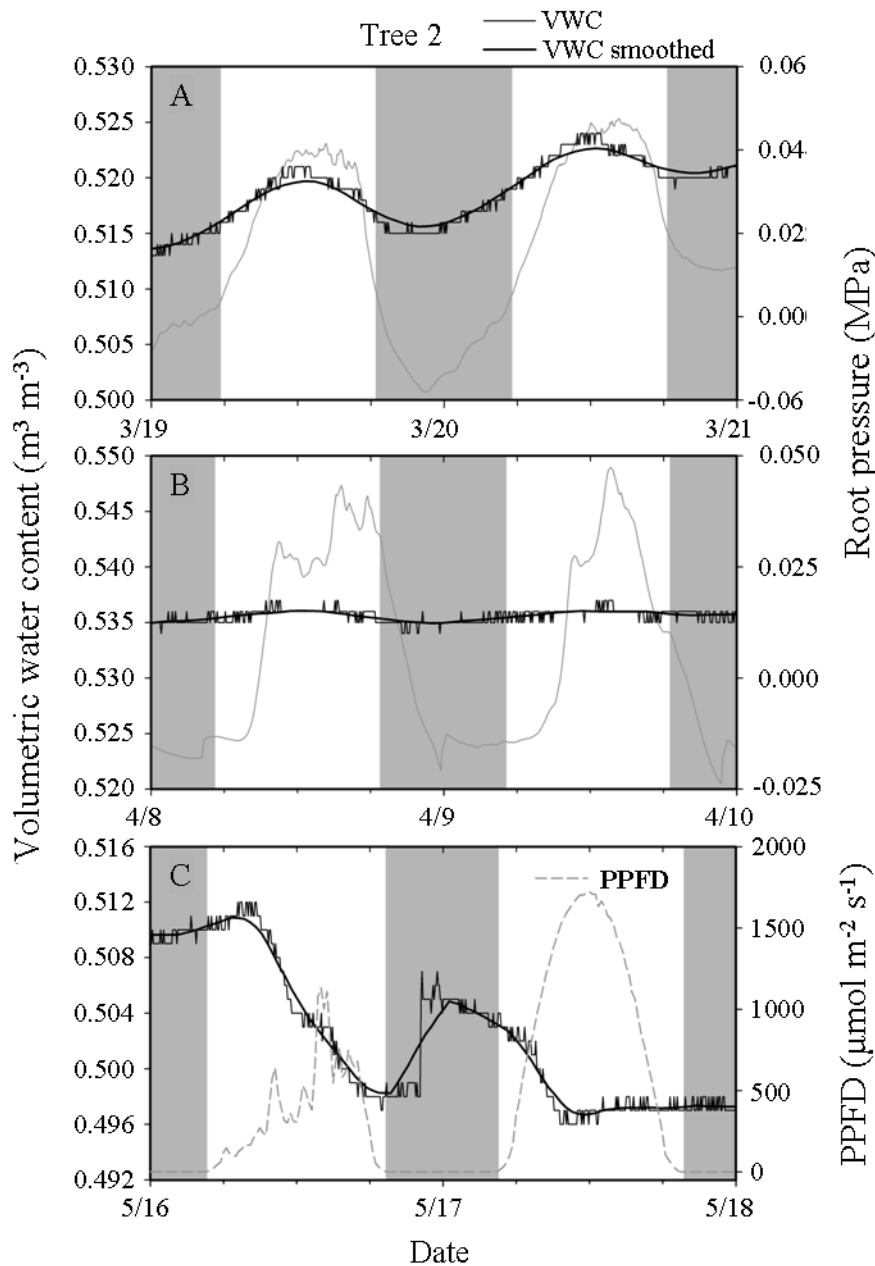
The apparatus used for the trunk air injection showing the FDR moisture sensor and a customized block designed to hold the PEEK tubing for high-pressure gas delivery. The DBH (diameter at breast height) of the tree is 18.7 cm.

Figure B.2



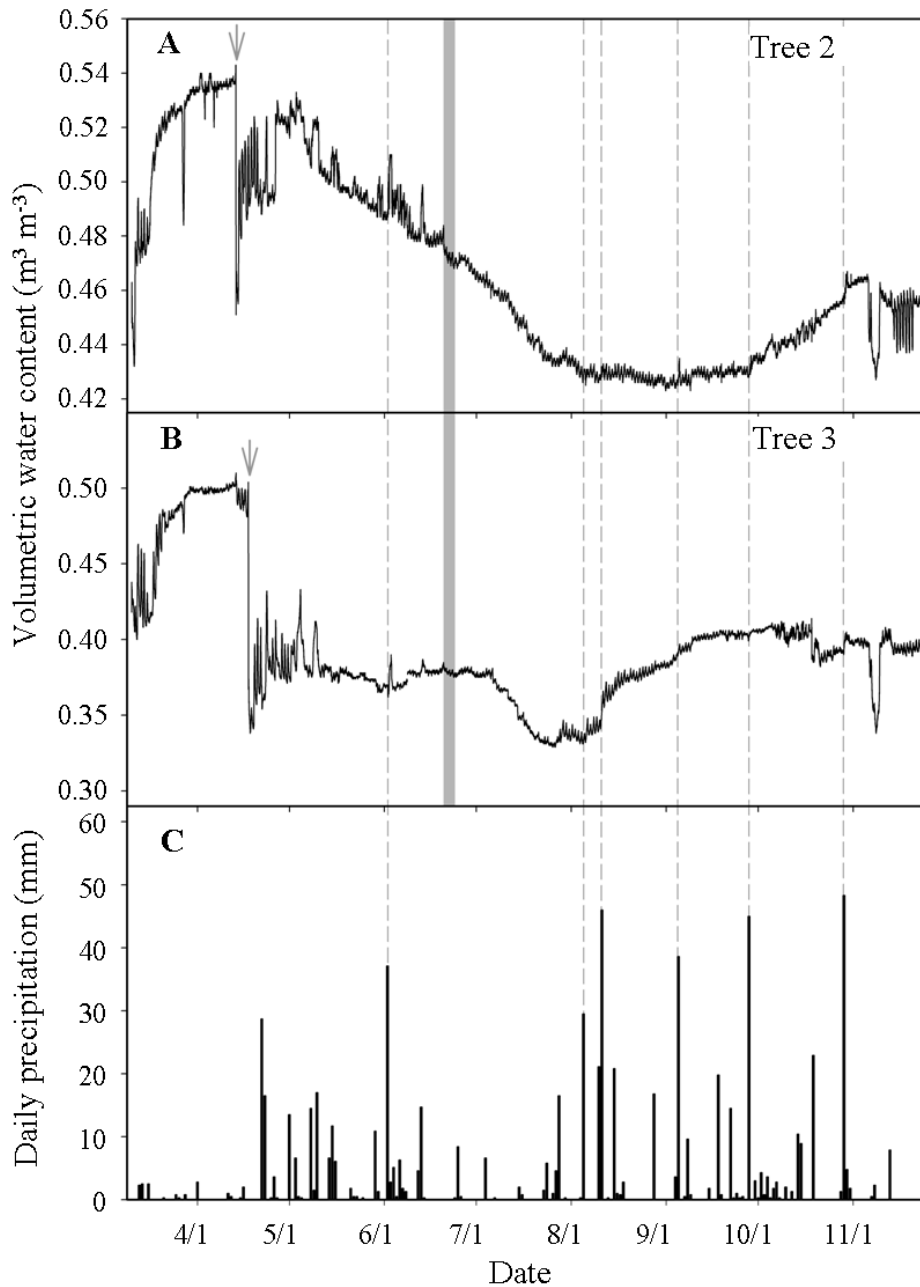
(A) FDR sensor installed in a *Betula papyrifera* wood block (ca. 10 cm \times 8 cm \times 3.5 cm) used for calibration in the laboratory; (B) volumetric water content (VWC) measured by FDR sensors plotted against VWC data calculated gravimetrically (data from two sets of independent calibrations were pooled). The dashed line show $y = x$; (C) tree core VWC measured gravimetrically vs. VWC measured using FDR sensors *in situ* on April 13, 2012. Error bars show ± 1 SE ($n = 5$).

Figure B.3



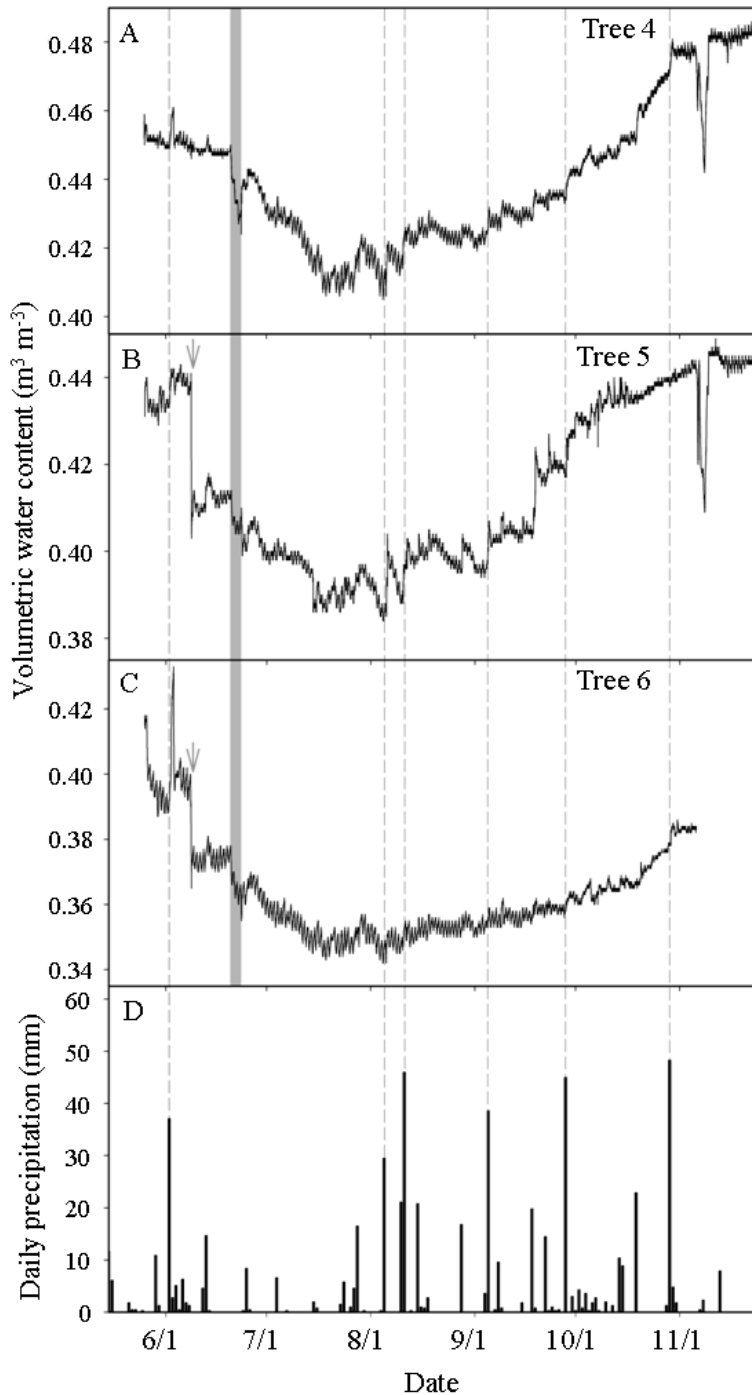
Simultaneous measurements of trunk volume water content (VWC) and root pressure (RP) in a representative tree in early spring showing: (A) the synchronized diurnal fluctuation of VWC with RP and overall increase of VWC; (B) a lack of significant diurnal change in VWC after it reached the maximum level irrespective of the diurnal root pressure fluctuation; (C) fluctuation of VWC after leaf expansion in the absence of root pressure. Dark bars denote nighttime periods. Note that higher VWC (in A) and RP (in A and B) occurred during daytime that peaked around midday but in panel C decreases in VWC were observed during the daytime due to transpiration.

Figure B.4



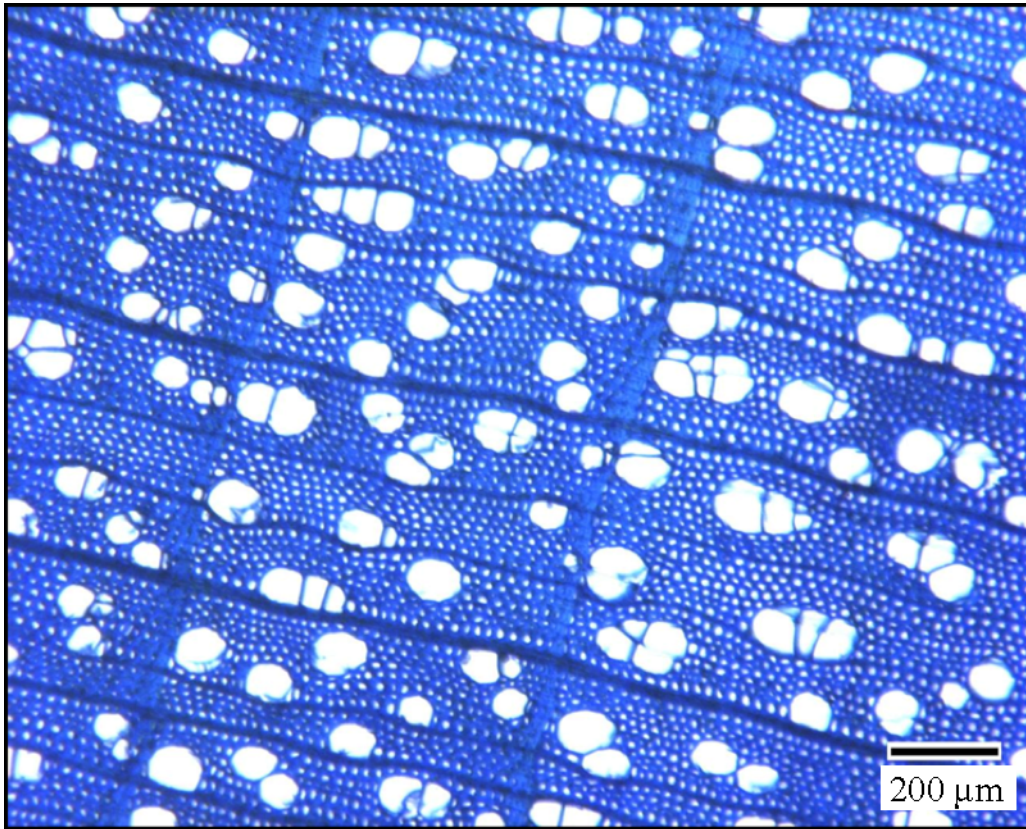
Seasonal change of volumetric water content (VWC) in trunk xylem of tree 2 (**A**) and tree 3 (**B**); (**C**) daily sums of precipitation. Arrows in panels A and B show air injection in early spring. Vertical dashed lines show days with significant rainfall, during which substantial increases in trunk xylem VWC were observed in most cases as shown in Figure 5.2A. The vertical gray bar in panels A and B denotes a substantial decrease in VWC during a summer heat wave shown in Figure 5.2B.

Figure B.5



Seasonal change of volumetric water content (VWC) in trunk xylem of tree 4 (A), tree 5 (B) and tree 6 (C); (D) daily sums of precipitation. Arrows in panels B and C show air injection in the summer. Vertical dashed lines show days with significant rainfall, during which substantial increases in trunk xylem VWC were observed in most cases as shown in Fig. 2A. The vertical gray bar in panels A-C denotes substantial decrease in VWC during a summer heat wave shown in Figure 5.2B.

Figure B.6



A cross-section of *B. papyrifera* trunk xylem showing the diffusely distributed vessels within densely arranged fibers and thin lines of ray parenchyma cells.