Axial anatomy of the leaf midrib provides new insights into the hydraulic architecture and cavitation patterns of *Acer pseudoplatanus* leaves

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Abstract

The structure of leaf veins is typically described by a hierarchical scheme (e.g. midrib, 1st order, 2nd order), which is used to predict variation in conduit diameter from one order to another whilst overlooking possible variation within the same order. We examined whether xylem conduit diameter changes within the same vein order, with resulting consequences for resistance to embolism. We measured the hydraulic diameter (*D*ₜ), and number of vessels (*Vₙ*) along the midrib and petioles of leaves of *Acer pseudoplatanus*, and estimated the leaf area supplied (*A*ₜₗₘₜₜ) at different points of the midrib and how variation in anatomical traits affected embolism resistance. We found that *D*ₜ scales with distance from the midrib tip (path length, *L*) with a power of 0.42, and that *Vₙ* scales with *A*ₜₘₜₜ with a power of 0.66. Total conductive area scales isometrically with *A*ₜₘₜₜ. Embolism events along the midrib occurred first in the basipetal part and then at the leaf tip where vessels are narrower. The distance from the midrib tip is a good predictor of the variation in vessel diameter along the 1st order veins in *A. pseudoplatanus* leaves and this anatomical pattern seems to have an effect on hydraulic integrity since wider vessels at the leaf base embolize first.

Keywords: *Acer pseudoplatanus*, Leaf cavitation, leaf hydraulic architecture, leaf midrib, total conductive area, sycamore maple, vessel diameter, vessel number.

Introduction

The leaf is the terminal part of the water transport system in plants and is the location of a substantial amount of hydraulic resistance (Sack and Holbrook, 2006). The physiological mechanisms and morphological traits of leaves largely determine how water deficit affects plants. The control of stomatal conductance induced by leaf dehydration is the primary mechanism involved in limitation of embolism events (Scoffoni et al., 2017a). However, during prolonged conditions of soil water shortage, the leaf veins are prone to embolism. Major veins are reported to be more vulnerable to embolism than minor veins (Brodribb et al., 2016a, 2016b; Scoffoni et al., 2017b) and, for angiosperms, the leaf vessel diameter is narrower in the minor veins compared to the major veins (Coomes et al., 2008; Carvalho et al., 2017; Scoffoni et al., 2017b). Conduit diameter is an important trait to evaluate hydraulic conductivity, and correlations between conduit diameter and vulnerability to embolism have been shown in both stems (Cai and Tyree, 2010; Olson et al., 2018; Liu et al., 2019) and leaves (Brodribb et al., 2016a, 2016b; Scoffoni et al., 2017b). Therefore, understanding how and why vessel diameters vary within the leaf is crucial for
predicting the efficiency of water transport and how embolism events might spread throughout the hydraulic network.

To study the hydraulic architecture in leaves, the vein network is usually divided following a hierarchical grouping of the leaf veins: the 1st order veins run from the base of the lamina to the leaf apex, the 2nd order veins branch from them, and the 3rd and higher orders decrease in diameter and form the reticulated network that transports water to close to the substomatal cavities (Fig. 1A) (Hickey, 1973; Ellis et al., 2009; Sack et al., 2012; Carvalho et al., 2017; Gleason et al., 2018). This hierarchical grouping has been used to test whether the leaf vascular system follows the structure proposed by Murray’s law. This law predicts that variation in conduit diameter occurs only among different vein orders, but not within the same order, and that the sum of all inner radii cubed at the level of one order is equal to the sum of all inner radii cubed at more distal orders (McCulloh et al., 2003, 2009; Gleason et al., 2018). Thus, the total conductive area across all vein orders increases from the 1st order vein (midrib) to the 2nd (and higher), but remains invariant within the same vein order (McCulloh et al., 2003, 2009; Gleason et al., 2018).

To our knowledge, there are few studies that have evaluated possible variations within the same vein order. A study on leaves of ash species by Petit and Anfodillo (2013) showed variation in conduit diameter along the leaf rachis, with vessel diameter widening from the apical leaflet base to the petiole, suggesting that vessel diameter scales with leaf length. Variation of conduit diameter with path length is theoretically predicted by the West, Brown, and Enquist (WBE) model for tree stems (West et al., 1999) and this has been supported by several studies conducted on different species (Anfodillo et al., 2006; Olson et al., 2014, 2018; Petit et al., 2014). The WBE model predicts that conduit widening from the top of the tree to its base is involved in compensating for the potential increase of hydraulic resistance imposed by increasing height (i.e. increase of path length, $L$) and that the conduit hydraulic diameter ($D_h$) should scale with $L$ with an exponent of $\approx 0.2$, approximately following a power law (West et al., 1999; Anfodillo et al., 2006):

$$D_h = a L^b$$

(1)

Where $a$ is the conduit diameter measured at the most distal position and the exponent $b$ is the degree of widening. The WBE model assumes that leaves are invariant terminal units that do not affect the scaling relationship of anatomical traits in the stem, such as conduit diameter and number. Although this assumption has aroused some controversy (Kozlowski and Konarzewski, 2004, 2005; Mäkelä and Valentine, 2006), the prediction of path length as the main driver for stem conduit diameter has been confirmed by several studies on different species and environments (Anfodillo et al., 2006, 2013; Lintunen et al., 2010; Olson et al., 2014, 2018).

The aim of this study was to examine whether leaf vessel diameter and vessel number scale with path length, or if vein order is the only predictor in leaf hydraulic architecture. In order to do this, we considered the midrib as the ‘stem’ and the distal part of the midrib close to the substomatal cavities at the leaf tip as the ‘terminal unit’. Finding variations in vessel diameter and number along the midrib would suggest that the prediction of the WBE model that vessel diameter scales with path length is also valid for the leaf. Alternatively, finding no variation in vessel diameter along the midrib would support the prediction of Murray’s law that assumes that changes in vessel diameter only occur between vein orders. Moreover, given that vessel diameter in veins is associated with hydraulic conductivity and susceptibility to embolism (Scoffoni et al., 2017b), the spread of embolism along the midrib should follow the pattern of vessel diameter. We tested two possible patterns. In the case of vessel diameter widening along the midrib, we would expect wider vessels at the base of the vein to embolize first and the narrow vessels at the midrib tip to embolize later. In the case of constant vessel diameter, we would expect embolism to initiate randomly along the midrib.

![Fig. 1.](image-url)
We also examined how total conductive area scales with leaf area along the midrib. Total conductive area is affected by the vessel lumen area (\(V_{\text{area}}\)) and the number of vessels (\(N\)). From the WBE model prediction we would expect that total conductive area (TCA) scales with leaf area (\(A_{\text{leaf}}\)) with an exponent >1. The WBE model predicts that there is a constant vessel number per unit of leaf area (\(V_N = A_{\text{leaf}}^{1.13}\)), that the vessel diameter widens with increased path length with an exponent of 0.2, without approximations for tree height (see Anfodillo et al., 2006; Olson et al., 2018), and that leaf area scales with path length with an exponent of 3 (West et al., 1999). Using these exponents, we calculate that vessel area (\(V_{\text{area}} = D_h^2\)) scales with leaf area with an exponent of 0.13 (\(V_{\text{area}} = A_{\text{leaf}}^{0.2 \times 2/3}\)). Summing the exponents of \(V_N = A_{\text{leaf}}^{1}\) and \(V_{\text{area}} = A_{\text{leaf}}^{0.13}\), the total conductive area is expected to scale with leaf area with an exponent of 1.13.

Murray's law predicts that the total conductive area across all vein orders increases from the 1st order vein (midrib) to the 2nd (and higher), but remains invariant within the same vein order (McCulloh et al., 2003, 2009; Gleason et al., 2018). Following the predictions of Murray's law, we would expect no variation of total conductive area along the midrib.

Material and methods

Plant material

We collected five apical shoots of young Acer pseudoplatanus L. plants growing in a wooded area of San Vito di Cadore (Northern of Italy). We chose A. pseudoplatanus for the study because it has leaves that can reach more than 15 cm in length and width (petiole excluded) at full development. The lamina is thin and the vein network is clearly visible, so it is possible to easily identify the pathways of individual minor veins. The length and the thickness of the lamina also allows several anatomical sections to be obtained along the midrib and the leaf area supplied for each section to be traced. Samples were collected at the end of the growing season (August 2016) to ensure that leaves were fully developed, and we selected leaves with similar exposure to light. The leaves were preserved in 50% alcohol. Seven healthy leaves were selected for the measurements. For each leaf we measured the leaf area (\(A_{\text{leaf}}\)) and prepared anatomical sections along the midrib (~10–16 depending on leaf size) (Fig. 1B, C).

Anatomical sections

Anatomical cross-sections were taken at different distances from the midrib tip. As is also observed for the stem, conduit widening is well described by a power law, meaning that the widening is greater at the apical part (West et al., 1999; Petit et al., 2008; Anfodillo et al., 2013). To examine whether vessel diameter and number along the midrib also changed steeply at the tip of the leaf, we prepared sections at 1-cm intervals starting from the distal tip of the vein for the first 3 cm, and then at 2-cm intervals for the rest of the leaf lamina and petiole (Fig. 1B). The samples were embedded in paraffin and sections were cut at 14 μm thickness using a RM 2125 rotary microtome (Leica Biosystems). We used a solution of safranin (1%) and astra blue (0.5%) to stain the sections to highlight the lignified cells and better distinguish the xylem vessels, and then permanently mounted them on slides using Eukitt (BiOptica, Milan, Italy). Slides were scanned with a D-Sight 2.0 scanner (A. Menarini Diagnostics, Florence, Italy) at 100× magnification, and images were analysed using the ROXAS software (www.wsl.ch/roxas). This software is widely used for analysing woody anatomical sections since it automatically measures a large number of "particles" (i.e. vessels or conduits) (Wegner et al., 2013; von Arx et al., 2016). The software counted the number of vessels (\(V_h\)), and calculated the total conductive area of the midrib section (TCA, calculated as the sum of the vessel areas) and the hydraulic weighted diameter (\(D_h\)). \(D_h\) accounts for the contribution of vessels to hydraulic conductance (Kolb and Sperry, 1999) and was calculated as the sum of the measured vessel diameters (\(d\)) to the 5th power divided by the sum of the vessel diameters to the 4th power:

\[
D_h = \frac{\Sigma d^5}{\Sigma d^4}
\]

where the diameter \(d\) is measured from the cross-sectional area of each vessel.

Leaf area (\(A_{\text{leaf}}\)) was obtained by scanning the leaf lamina (1000 dpi) and analysing the image using ImageJ (https://imagej.nih.gov/ij/). The high-quality scans allowed estimation of the portion of leaf area supplied by the vessels at different distances from the leaf tip, as follows. On the scans, we traced the points where the anatomical sections were obtained along the midrib. Starting from the leaf tip, we traced the sector of leaf area supplied by the vessels at 1 cm from the midrib tip. We accounted for the contribution of the 2nd and 3rd order veins in suppling water to each leaf sector (Fig. 1B, C). We summed the area sectors from the leaf tip to the petiole to obtain the leaf area supplied (\(A_{\text{leaf-sup}}\)) for each anatomical section. For this estimation, we assumed that the water flow was unidirectional from the base to the tip of the lamina and that the distal part of the midrib serves only the distal part of the leaf lamina. We estimated the number of vessels per unit of leaf area (\(V/cm^2\)) along the midrib as the number in the anatomical cross-section divided by the supplied leaf area.

Vulnerability to embolism

Three small healthy saplings of A. pseudoplatanus were cut at the base of the stem at the level of the root collar just before dawn. They were immediately placed in black plastic bags with damp paper towels to reduce water loss and transported to the laboratory.

We selected one healthy leaf from each sapling and used the optical method described by Brodribb et al. (2016a, 2016b) to identify embolism events in the midrib. Images were taken using a reflex camera every minute for 24 h. Due to the thickness of the midrib we performed the measurements using reflected light (see http://www.opensequence.org/overview/the-optical-method/). The image sequences were analysed using ImageJ to identify embolism events, seen as changes in the reflectance of the midrib. We used the toolbox ‘OSOV’ (https://github.com/OpenSourceOV) to perform image subtraction of subsequent images to reveal rapid changes in light reflectance. Embolism events were assigned a threshold, allowing automated counting of each event using the ‘analyse-stack’ function. Noise was removed using outlier removal. A map of embolism events, colour-coded for time, was automatically generated by colouring the embolism area in each sequence, with the first event of cavitation at time 0.

Statistical analysis

Data were log10-transformed in order to meet normality and homoscedasticity assumptions. We used the lmef2 package in R (version 3.3.1, www.r-project.org) to perform type-II linear regressions with reduced major axis (RMA), which is appropriate for studying allometric relationships between co-dependent variables (Smith, 2009). We examined whether \(D_h\) and vessel number varied with distance from the leaf tip (i.e. path length, L). We also looked at how the number of vessels scaled with the leaf area supplied (\(A_{\text{leaf-sup}}\)) and whether there was variation in the number of vessels per unit area from the tip to the base of the midrib.

In addition, we examined how the total conductive area scaled with \(A_{\text{leaf}}\), and whether the exponent obtained differed from 1.16 as predicted by the WBE model.

Results

The anatomical traits of the xylem varied markedly along the midrib. For the leaves that we sampled, the area ranged between...
82–217 cm² and midrib length between 9–15 cm. At the distal part of the midrib, the xylem was composed of narrow vessels with a high number per unit of area (V/cm²). Moving from the leaf tip to the base of the petiole, the general observed pattern was that vessels widened in hydraulic weighted diameter (Fig. 2) and decreased in number per unit leaf area (Fig. 3). No major changes in anatomical traits were observed at the junction between the leaf lamina and the petiole.

Dₜ widened considerably from the midrib tip (5.5–6.5 µm) to the base of the petiole (20–25 µm), an increase of ~4-fold. The relationship between Dₜ and the path length, L, was well described by a power law, with an exponent of 0.42 (R²=0.88; Table 1) (Fig. 2).

The number of vessels increased from the midrib tip (15–40 at 1 cm from the tip) to the midrib base and along the petiole (between 190–383), V₅ scaled with Aₖₜₜ₂ with an exponent of 0.66 (Fig. 3A), which was significantly less than 1 (R²=0.92; Table 1). Vessel number per unit of leaf area supplied varied along the midrib, with the highest values occurring close to the leaf tip (>50 cm⁻²) and decreasing sharply towards the leaf base (<12 cm⁻²), reaching a plateau along the petiole (Fig. 3B).

The total conductive area (TCA, calculated as the sum of all vessel areas) scaled isometrically with the leaf area supplied (Fig. 4) with an exponent of 0.98, which was not significantly different from 1 (R²=0.97; Table 1) but was statistically different from 1.13, the value predicted by the WBE model.

The first cavitation event always occurred in the basal part of the midrib at about 7–10 h after the sapling was excised (Fig. 5). Cavitation at the leaf tip occurred ~2.5–3.3 h (150–200 min) later, and continued for about 6–7 h (500–540 min).

Discussion

In this study, we examined how anatomical traits of vessels in the leaves of Acer pseudoplatanus varied with midrib length and how embolism spread along the midrib. We demonstrated that midrib length allows the prediction of traits such as vessel diameter, vessel number, and total conductive area. We also found new evidence supporting the idea that wider vessels are more vulnerable to embolism than narrow ones, with our results showing that embolism events within the midrib occur first at the base where the conduits are wider.

The prediction of Murray’s law that variation in vessel diameter should occur only among vein orders but not within the same order was not confirmed by our results. Vessel hydraulic weighted diameter varied significantly along the midrib of the leaves (Fig. 1). Indeed, it increased along the midrib and petiole with a degree of widening more than double that reported for the stem xylem (0.42 versus 0.2) (Anfodillo et al., 2006; Petit et al., 2008; Olson et al., 2014), but similar to that reported for leaves of ash (Petit and Anfodillo, 2013). Our results showed that, in common with the stem, leaf vessel diameter scaled with the distance from the apical part within the same vein order, and the widening exponent was higher than has previously been reported for the stem.

Basipetal widening of vessels is consistent with the prediction of equal flow to all the possible paths. In the case of a tree, this means that leaves located at different heights within the crown will be supplied uniformly (West et al., 1999). Similarly, in the leaf, stomata are located at different distances from the
midrib base and the tip–to-base widening of the vessels along the midrib is probably involved in buffering the increase in hydraulic resistance due to the path length across the leaf lamina.

The vessel diameter in the distal part of the midrib (1 cm from the tip) was similar to that reported for the minor vein vessels of *Populus* species, where the diameter in the 7th order veins was ~5±1 µm (±SE) (Carvalho et al., 2017). This is in line with the intercept value of 5.75 µm (CI: 5.37, 6.16) calculated from the $D_h$ versus $L$ regression (Fig. 1). Natural selection may have favoured narrow vessels in the terminal parts of the transport system because they maximize the diffusion surface per volume of water. It is then possible that vessel diameters in the proximity of the terminal parts of the water transport system, where the it diffuses into the mesophyll, might be similar among different species.

Recent studies have shown that wider vessels are more vulnerable to embolism than narrow ones (Scoffoni et al., 2017b; Olson et al., 2018; Liu et al., 2019). Olson et al. (2018) showed that taller plants with wider vessels in the stem are more vulnerable to embolism than shorter conspecifics that have smaller vessels, and Gleason et al. (2018) found taller species to be more vulnerable to embolism than shorter ones. In leaves of different species, Scoffoni et al. (2017b) found that embolism occurred first in the wider vessels of the petiole and midrib, followed by the vessels in the 2nd order veins andLastly in the minor vein vessels. Our results showed that embolism initiated at the base of the midrib (Fig. 5) where the vessel hydraulic diameter was wider, supporting the idea that wider vessels are more vulnerable than narrow ones. Brodribb et al. (2016a) also showed that 1st order veins (i.e. the midrib) are the first to embolize and that the time interval between the first and last embolism event within a vein order is greater in longer veins. Based on our results, we would suggest that longer veins have a greater difference in vessel diameter from the tip to the base. Long veins would embolize at different times, first at the base where vessels are wider, and then at the tip where they are narrower. In short veins, where the difference in vessel diameter from tip to base is less, the time interval between the first and last embolism events would be shorter.

Vessel number ($V_N$) and vessel number per unit of leaf area supplied ($V_{leaf-sup}$) also varied along the midrib from tip to base. At the tip, vessels were narrow with a high number per leaf area, and they widened and decreased in number towards the base (Fig. 3). Variation in vessel diameter and number may affect hydraulic conductivity along the midrib. The Hagen–Poiseuille law predicts that hydraulic conductivity depends mainly on diameter and length of the conduits, and a small increase in diameter corresponds to a great increase in conductance (Tyree and Zimmermann, 2002). If a narrow conduit is favoured by selection at the terminal part of the transport system to maximize the water diffusion surface (West et al., 1997, 1999; Sack et al., 2012), then this also results in a decrease in its conductivity. An increase in vessel number per unit of leaf area at the tip of the midrib may favour conductivity to the substomatal cavities.

The combination of a high degree of widening and a decrease in vessel number per unit area from the leaf tip to the base of the petiole resulted in an isometric relationship between the total conductive area and leaf area supplied (Fig. 4).

The exponent of this relationship was significantly different from the one predicted by the WBE model ($TCA \propto A_{leaf}^{1.13}$). Isometry between TCA and leaf area is a trait found in stems, branches, and leaves (Togashi et al., 2015; Fan et al., 2017; Petit et al., 2018), and the relationship between these two traits is used to understand the relationship between hydraulic capacity and photosynthesis (Petit et al., 2018).

One of the most important predictions of the WBE model is that leaf specific conductance, i.e. water flow per unit of leaf area, is invariant and independent of leaf position in the crown or, in other words, independent of the conductive path length or the tree size. This prediction can be tested by comparing the scaling exponents of the relationship between the theoretical flow rate ($Q_{th}$) and leaf area resulting from our study and the exponents predicted by the WBE model. Theoretical flow rate can be estimated as the vessel hydraulic diameter at the 4th power multiplied by number of vessels ($Q_{th} = D_h^{4} \times V_N$) (Fan et al., 2017). From our results, vessel diameter scaled with leaf area as $D_h \propto A_{leaf-sup}^{0.19}$ and vessel number scaled with leaf area supplied as $V_N \propto A_{leaf-sup}^{0.66}$ (Table 1). The theoretical flow rate would then scale with leaf area supplied as $Q_{th} \propto (A_{leaf-sup}^{0.19}) \times A_{leaf-sup}^{0.66}$, resulting in $Q_{th} \propto A_{leaf-sup}^{1.42}$. Using the exponents predicted by the WBE model and given that the leaves that we measured had a relationship of $A_{leaf} \propto L^{2.13}$ (data not

| $a$ [CI] | $b$ [CI] | $R^2$ | $P$-value |
|----------|----------|-------|------------|
| $D_h$ versus $L$ | 0.76 [0.73, 0.79] | 0.42 [0.39, 0.45] | 0.88 | <0.0001 |
| $D_h$ versus $A_{leaf-sup}$ | 0.89 [0.86, 0.92] | 0.19 [0.17, 0.20] | 0.87 | <0.0001 |
| $V_N$ versus $A_{leaf-sup}$ | 1.44 [1.38, 1.50] | 0.66 [0.61, 0.70] | 0.92 | <0.0001 |
| TCA versus $A_{leaf-sup}$ | 2.97 [2.92, 3.02] | 0.98 [0.94, 1.03] | 0.97 | <0.0001 |

$a$, $y$-intercept; $b$, slope; CI, 95% confidence intervals; $D_h$, hydraulic diameter of the conduits; $V_N$, number of vessels; TCA, total conductive area; $L$, path length (distance from leaf tip); $A_{leaf-sup}$, leaf area supplied. Data are log$_{10}$-transformed.
shown), then $D_h$ should scale with $A_{leaf-sup}$ with an exponent of $\sim 0.1$ and vessel number should scale isometrically with leaf area ($V_N \propto A_{leaf}^{1/4 \times A_{leaf-sup}^{1}}$, resulting in $Q_{th} \propto A_{leaf-sup}^{1.4}$. Despite the exponents found for $D_h \propto L$ and $V_N \propto A_{leaf-sup}$ being significantly different from those predicted by the WBE model, the exponent for $Q_{th} \propto A_{leaf-sup}$ is nearly identical to the predicted one, suggesting that model prediction of independent leaf specific conductance can also be considered valid for the leaf hydraulic architecture.

We only considered one vein order in this study and hence we cannot exclude the possibility that variation among vein orders follows the predictions of Murray’s law, but we suggest that the position along the vein at which anatomical traits are measured has to be taken into account. Our results showed that vessel diameter and vessel number scaled with the length of the midrib, and the exponent that we found for vessel widening in the leaf was double that predicted by the WBE model for the stem. The WBE model predicts that specific conductance is independent of path length. Despite the differences that we found in the predicted exponents, the model might still be valid for describing the leaf hydraulic architecture, but only through using a different combination of scaling exponents. Further studies are needed to evaluate whether this is consistent between different species and also whether it is possible to identify universal hydraulic architectures in leaves.

Finally, we can conclude that anatomical traits of the midrib vary with the distance from the terminal part of the water transport system, and that wider vessels along the midrib are more vulnerable to embolism than narrower ones. We suggest that leaf length and/or leaf area supplied are key traits to be considered in leaf architectural models.

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