# Measuring vessel length in vascular plants: can we divine the truth? History, theory, methods, and contrasting models 

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#### Abstract

Key message The Cohen method of measuring vessellength distributions is much more accurate than the DD algorithm on integer values, which should be abandoned. More research is needed to get the real distribution of vessel length. Abstract Scientists have been measuring the vessel length of plants for more than 50 years. The method involves infusing stem or segments with a visible substance that completely fills vessels cut open at the infusion surface. The number of infused vessels is then quantified versus distance from the infusion surface. A theoretical model is then used to convert the counts of infused vessels to a vessel length distribution. Over the years the methods and theory have changed greatly. The purpose of this review is to give the reader an understanding of why vessel length is important and to provide a theoretical basis for selection of the best method and theory to arrive at vessel length data.


Keywords Vessel length • Vessel length distribution • Efficiency versus safety • Cohesion-tension theory

Vessel lengths are difficult to visualize in a microscope because they are small enough in diameter to require a microscope to see them (mostly $0.01-0.2 \mathrm{~mm}$ ) but the microscope is too myopic to see more than $0.1-1 \mathrm{~mm}$ of their full length $\left(10-10^{4} \mathrm{~mm}\right)$. Their small diameter-tolength ratio also makes it impossible to represent them in

[^0]scale drawings. Plant scientists have been interested in vessel length for more than 60 years. Greenidge (1952) talked about maximum vessel length and Scholander (1958) talked about 'average' vessel length. But the concept of vessel-length distribution originated with Skene and Balodis (1968). After 1968 over 150 papers have reported measurements of vessel length and over 800 refer to values of vessel length. In studies before 2000 most papers report vessel length distributions from the perspective of scientific curiosity. However, there is now a more fundamental reason for wanting this information. Vessel length is needed to evaluate the trade-off between hydraulic efficiency of vessels versus safety against cavitation. Vessel length is tied to the unique and daring means by which land plants transport water from the soil to the leaves.

## Evolution of vascularization in plants

The evolution of vascularization in plants was driven by the same physical constraints as in animals; vascularization is needed for the rapid movement of water and nutrients over long distances because diffusion is too slow to sustain life in large organisms without a pathway for mass flow. However, there are two fundamental differences between plant and animal vessels: (1) the lumina in animal vessels are surrounded by a wall comprised of living cells whereas the lumen of a plant vessel is the dead remains of an individual cell with only the cellulose cell wall remaining after death. (2) Fluid transport in animal vessels is under positive pressure whereas in plants fluid is transported under negative pressure and hence fluid is transported in a metastable state (see below).

Primitive vascular land plants transport water in tracheids. In conifers, tracheids divide from cambial cells located between the bark and wood of stems. Overlapping
files of cells divide and enlarge to quite long cylindrical cells $10-50 \mu \mathrm{~m}$ in diameter $(D)$ and $1,000-7,000 \mu \mathrm{~m}$ in length $(L)$, (Fig. 1e, f, g). To become functional these cells must die, but before they die they differentiate elaborate connections between overlapping cells called bordered pits (Fig. 2). Water flowing through a tracheid lumen encounters a hydraulic resistance to water flow that approximately equals the hydraulic resistance of the pits. Lancashire and Ennos (2002) proposed a theory that the optimum wood conductance occurs when the tracheid length increases as a defined function of diameter because lumen resistance changes proportional to $L / D^{4}$ Whereas pit resistance changes proportional to the lumen surface area $\alpha L D$ where $\alpha=\pi \times$ (fraction of surface occupied by pits).

Vessels are composed of linear files of vessel elements. Each element starts as a living cell and dies to become functional. Prior to death the end walls of vessel elements in series partly or fully disappear to form perforation plates between the many vessel elements to make one long vessel. Tracheids are thought to have evolved into vessel elements by becoming larger in diameter (up to $400 \mu \mathrm{~m}$ ) but shorter than tracheids (Fig. 1h, i, j, k); however, vessels can consist of hundreds to thousands of vessel elements resulting in vessel lengths anywhere from 0.001 to $>10 \mathrm{~m}$ long. Conifer tracheids also evolved into shorter and smaller diameter cells (called wood fibers) that pack around vessels to increase the mechanical strength of angiosperm wood (Fig. 1a, b, c, d). Like tracheids, individual vessels are interconnected with other vessels when they occur in vessel clusters. Usually vessels are solitary in wood surrounded by smaller living or dead cells, but tend to wander around within the wood volume to make occasional contact with other vessels (Fig. 3) as they follow the main axis of stems. Water passes from vessel to vessel through pit fields where adjacent vessels adjoin (Fig. 3). Alternatively, if solitary vessels are surrounded by fiber tracheids, then water can flow between vessels via fiber bridges (Cai et al. 2014). Tyree and Zimmermann (2002) have suggested that in rattan vines, where very long vessels ( $>5 \mathrm{~m}$ ) are surrounded by living parenchyma cells, water can pass efficiently via parenchyma cell membranes.

Tracheids are easily measured by a maceration of wood in strong acid, which causes the tracheids to dissociate into individual cells allowing easy measurement of $L$ and $D$. In contrast, maceration of angiosperm wood dissociates vessels into individual vessel elements and hence more elaborate techniques need to be devised to estimate the length of vessels.

## Why vessel length matters: metastable water and the presumed trade-off

Vessel-lumen hydraulic resistance is approximately equal to the vessel-to-vessel resistance even in species with quite
long vessels. Hence average vessel length increases with vessel diameter to maintain the partitioning of hydraulic resistance between lumina and end walls (Hacke et al. 2006). The Hagen-Poiseulle Law predicts that the stem-hydraulic-conductance should increase with the square of vessel diameter (see Chapter 1 in Tyree and Zimmermann 2002) when conduits are packed into tight geometric arrays. So why don't plants have only large diameter vessels that run their entire length, from roots to leaves? Large vessels would be very efficient for transporting water because there would be little pressure drop along the root/ stem pathway, which in turn would make the water potential of leaves nearly that of soils. Photosynthesis rate increases with stem hydraulic conductance (Hubbard et al. 2001); hence large vessels would promote carbon gain, but large vessels are also more prone to cavitation (Cai and Tyree 2010) which reduces hydraulic conductance and decouples leaves from soil water reserves. That is the basis of the presumed trade-off.

The reason plants tend to have shorter-than-possible vessels with smaller-than-optimum diameters has a lot to do with the daring means by which plants transport water. The cohesion-tension theory says that plants transport metastable water, i.e., water under negative pressure $=$ less than atmospheric pressure. The cohesion-tension theory has been validated indirectly by many measurements (Tyree 1997) as well as by the direct measurement of negative pressure in maize and woody vines (Wei et al. 1999). cohesion-tension theory explains why stems have complex networks of tracheids or vessels. The cohesion-tension theory also explains why vessels are connected to each other via pit membranes. These elaborate structures are needed to transport water in plants safely.

How does vessel structure work to allow for the daring transport of metastable water? A vessel interconnected with adjacent vessels allows for redundancy in the pathway of water movement and protects plants against the frequent instances in which metastable water breaks down by cavitation. When tension (negative pressure) becomes too large, an air-bubble is rapidly sucked into a water-filled vessel or tracheid (cavitation), rapidly expands to fill the entire vessel with a partial vacuum, and then air comes out of solution in surrounding tissue to form an air bubble (an embolism) at atmospheric pressure. An embolized vessel is incapable of transporting water and typically remains embolized for many hours or forever if the plant has no mechanism to refill embolized vessels. The pit-membranes between adjacent vessels usually function to prevent the propagation of air from an embolized vessel to an adjacent vessel filled with metastable water. The surface tension of water prevents the air/water interface from passing through the pit membrane provided the pressure difference between the air-filled and water-filled sides of the membrane is not

Fig. 1 Tracheids in conifer wood evolved in two directions to become vessel elements and fibers in angiosperm wood. As illustrated, vessel elements tend to be larger in diameter than tracheids and are terminated by perforation plates that can be simple ( $K$ : a large hole) to scalariform ( $H$ : elongated holes separated by cellulose bands). Wood fibers are small linear cells packed around vessels and provide mechanical strength to angiosperm wood. Reproduced from (Bailey and Tupper 1918)

Fig. 2 Shown is a diagrammatic representation of tracheids in conifer wood.
$R=$ living ray cells,
$T=$ tracheid lumen, $P=$ pits, arrows indicate the direction of water flow through and between tracheids. Reproduced from Tyree and Zimmermann (2002)

too large. The pressure difference that can be sustained is approximately equal to $\Delta P=P_{\text {air }}-P_{\text {water }}=-2 \tau / r$, where $\tau=$ the surface tension of water and $r=$ the radius of the pore in the pit membrane. Based on the $\Delta P$ that induces embolism we suspect the pit membrane pores are $10-100 \mathrm{~nm}$ in diameter. The pit membrane with the largest pore in any given vessel lumen is thought to determine the tension at which it is likely to cavitate given the presence of an embolism in an adjacent vessel (see the air-seeding hypothesis in Sperry and Tyree 1988; Cochard et al. 1992). Readers interested in learning more are directed to the outstanding review on the theory of optimal vessel length published by Comstock and Sperry (2000).

In conclusion, water transport is divided between millions of independent vessels connected to each other in redundant pathways. Redundancy of pathways allows large numbers of vessels to cavitate without reducing transport efficiency enough to negatively impact tree performance.

## A brief history of methods

There are two accurate ways to obtain information on vessel length distribution but one is very labor-intensive (the cinematographic technique, Zimmermann 1971, 1978) and the other requires very expensive equipment (highresolution computer-aided X-ray tomography, Brodersen
et al. 2011). Hence the preferred approach is to (1) visualize the length of cut-open vessels by injecting vessels at the cut surface with some easily observed substance (colored or florescent) and (2) use a computational algorithm to deduce vessel length distributions based on a few assumptions.

## Perfusion

The trick is to perfuse the vessels with a colored substance that will pass through the vessel lumina and perforation plates without plugging but not pass through pit membranes. Vessel lengths can be measured in any plant organ but we will refer generally to 'stems' with the understanding that stem can also mean root or petiole too. Once a stem has been perfused with a colored substance it has to be stabilized in place and then the stem has to be sectioned into pieces of known length to observe how far the substance has traveled before reaching the vessel end.

The substance of choice used to visualize cut-open vessels has changed with time. Skene amd Balodis (1968) used oil-based paint. The paint pigment made the oil visible. When oil is injected (infused) into hydrated stems through a cut surface, the oil displaces water and the oil travels only as far as the end of the vessel because the surface tension of the oil/water interface prevents it from passing through pit membranes, provided the perfusion pressure is low enough.

Fig. 3 a A schematic representation of how vesselelements are arranged in single file to form vessels. Sometimes vessels are solitary and other times they are adjacent. $X$ indicates a small patch of wall where vessels adjoin. This area, which contains pits, is enlarged to the right and shown in cross section and face-on views. Reproduced from Zimmermann and McDonough 1978. b Actual arrangement of vessels in the wood of Cedrela fissilis. This scale drawing was constructed from detailed analysis of hundreds of serial section of the wood. Note that the vertical dimension is compressed about 10 times versus the other two dimensions. Numbers indicate vessel numbers. Reproduced from Zimmermann and Brown 1971



Vessels perfused with colorant must have no previous embolisms, because air bubbles cannot pass through pit membranes at the normal perfusion pressure. Hence, if a colorant is injected in front of a bubble, the colored liquid will compress the air bubble and travel only part way down the vessel until the pressure of the colored fluid equals the pressure of the compressed bubble. It is advisable to flush stems with degassed, clean water to remove embolisms before injecting colored solutions.

The colorant has to be composed of particles small enough to pass through perforation plates but too large to
pass through pit membranes (the ideal particle size is probably between 0.1 and $1 \mu \mathrm{~m}$ ). Later, oil-paint was replaced with water-based latex-paint diluted $100: 1$ or more in water; others have used the mineralized pigment particles used in latex-paint. Although many researchers have perfused stems with dilute latex-paint it is not advisable to use latex at all. Latex has little or no color and consists of amorphous particles that are sticky and tend to aggregate around the pigment particles and sticks to surfaces. That is why latex paint sticks so well to walls. It is far better to use the paint pigments without latex.

The mineralized pigments are fine enough to remain in suspension in water for long periods of time. Even though the colorant particles might be small, the particles tend to cluster into larger globules that will not pass through perforation plates. Premature plugging of vessels with particles before they have reached the vessel ends presents problems with subsequent analysis of data, i.e., causing an exclusion of the smaller diameter vessels (Ewers and Fisher 1989) or underestimating the length of long vessels. Consequently, the current technique is to inject vessels with silicone rubber recently mixed, but not yet polymerized (Andre 1998; Sperry et al. 2005).

Silicone rubber mixtures have been used extensively to visualize the nano-structures on the inner surface of vessels and are capable of moving the entire length of vessels if not impeded by air bubbles trapped in the vessels. Stem segments can be injected with silicone rubber using the technique described in Sperry et al. (2005) and Wheeler et al. (2005). Briefly, silicone rubber is freshly mixed from liquid silicone and hardener in the ratio of 10:1 ( 10 g RTV141 part A plus 1 g RTV141 part B) (Rhodorsil RTV-141; Rhodia USA, Cranbury, NJ, USA; imported by Walco Materials, Escondido, CA, USA). Uvitex, a fluorescent whitening agent (Ciba Uvitex OB; Ciba Specialty Chemicals, Tarrytown, NY, USA), is added to make the silicone visible under UV light. The Uvitex is dissolved in chloroform ( $1 \% \mathrm{w} / \mathrm{w}$ ) and 0.5 ml added to the silicone mix (Hacke et al. 2007). Uvitex does not move beyond the silicone rubber into wet wood because it is not water soluble. Stem segments are first flushed with a salt solution filtered to $0.2 \mu \mathrm{~m}$ at $0.05-0.15 \mathrm{MPa}$ for 30 min in order to remove air bubbles that would interfere with the injection of silicone rubber solution. After the water flush, the stems are injected with silicone at 0.12 MPa for 24 h , and then the silicone is allowed to cure (harden) for 3 more days at room temperature $\left(22^{\circ} \mathrm{C}\right)$ or 12 h in an oven at $38^{\circ} \mathrm{C}$ prior to sectioning. The cured stems are cross sectioned at several distances from the injection surface, and photographs are taken for vessel diameter measurement and count.

Once injected and polymerized, the vessel walls can be dissolved with strong acids leaving rubber micro casts of the interior surface of vessels. When the micro casts are viewed under SEM microscopy it is evident that the rubber passes through pit pore apertures up to but not through the pit membrane (Fig. 4); hence it is an ideal substance for injection with an aim toward computing vessel-length distributions (Andre 2002, 2005). However, for vessellength measurement, the stems are usually sectioned for further analysis without acid treatment. In cross section the vessels can be seen under a light microscope and the addition of some UV light will make the silicone shine brightly in rubber-filled vessels, making them distinct from vessels filled with water or air.


Fig. $4 \mathbf{a}$ and $\mathbf{b}$ Theoretical stem segments containing randomly distributed vessels (as defined in the text). Vertical bars indicate vessel ends. Hatched area indicates extent of paint-filled vessels infused from distance 0 . c Count of paint-filled vessels versus distance for short vessels (open circles) and long vessels (open squares). d Output of DD algorithm for theoretical stem $\mathbf{b}$ above. Vessels of different length are randomly distributed through the cross section of wood, but they are arranged in decreasing order of size for clarity without any loss of generality for the DD algorithm

Analysis methods

Once an adequate perfusion technique for a marker substance is in place, the analysis involves counting the number of vessels filled with the marker substance at cut surfaces and at various distances away from the injection surface. The vessel count, $N$, can be the total colored vessels in a stem or the number per unit area of wood cross section. The analysis algorithm assumes that vessels begin and end randomly over the length of the stem. Skene and

Balodis (1968) provide formulas to compute vessel-length distributions from a paint-infusion experiment. Later, Zimmermann and Jeje (1981) simplified the analysis equations into a tabular computational algorithm, i.e., the double-difference (DD) algorithm explained in more detail in Zimmermann (1983). The DD algorithm is used to convert the counts of paint-filled vessels into a frequency distribution (or probability density function, PDF) of the number of vessels in size classes of length $L$.

The primary difference between the Skene and Balodis (1968) analysis and the Zimmermann (1983) analysis is that the former plotted counts of colored vessels, $N$, versus distance, $x$, fit the plot to a smoothed function $(N(x)=f(x))$ and used interpolated decimal values to compute vessellength distributions, whereas Zimmermann (1983) used the raw integer values. Zimmermann (1983) later provided an easily comprehended visual illustration of how the DD algorithm works and why it is correct.

The DD algorithm is basically a computation of the second derivative times distance in finite steps using integer values (=count of paint-filled vessels). Zimmermann demonstrated the method with a simple example of a stem filled with vessels in two size classes (Fig. 4), wherein the vessel ends occur "randomly" (arranged in Fig. 4 in an orderly way which aids visualization without a loss of generality). In this example the stem is cut into 20 segments of equal length $(2 \mathrm{~cm})$ and counts made at the injection surface $(x=0)$ and at each cut-segment surface $(2,4,6 \ldots \mathrm{~cm})$. In the DD algorithm, the first difference between adjacent counts is computed and then the difference of the difference (Table 1) (=a second derivative of integer steps). The result is multiplied by the distance, $x$, from the injection surface (or the line number in Table 1 times segment length $=$ distance from injection surface) to yield the number of cells in each size class divided by $\Delta x$. As seen in Fig. 4 the reconstruction of size classes is quite precise if there are no 'errors'. One error not previously addressed is the clustering of random ends. In Fig. 4 the distribution of vessel ends was described as 'random' but in fact is 'equally spaced' which is not the same as random. The consequences of unequal spacing for the DD algorithm will be treated later but for now we will continue with the history.

The DD algorithm frequently provides PDFs that are 'unreasonable' because the algorithm results in some computed size classes that are of zero or negative probability preceded and followed by other size classes that are more reasonable positive numbers. In theory the negative numbers could result from vessels ending more frequently in specific regions of stems, e.g., near nodes or near vascular insertions connecting stems to petioles. Zimmermann and Jeje (1981) explained in detail how this could happen and how it can be corrected by subtracting negative values from

Table 1 The computations below illustrate the double difference (DD) algorithm (Tyree and Zimmermann 2002) for integer data as shown in Fig. 4, but it can also be used for decimal numbers as in Fig. 9 based on a regression fit

| Line no. | $x(\mathrm{~cm})$ | $N$ | First <br> difference $\begin{aligned} & \mathrm{N}^{\prime}=\Delta N / \\ & \Delta x \end{aligned}$ | Second difference $\Delta N^{\prime} / \Delta x$ | $x$ (Second difference) $x \Delta N^{\prime} / \Delta x$ | \% |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| 1 | 2 | 20 | $-1.5$ | 0 | 0 | 0 |
| 2 | 4 | 17 | $-1.5$ | 0 | 0 | 0 |
| 3 | 6 | 14 | $-1.5$ | 0 | 0 | 0 |
| 4 | 8 | 11 | $-1.5$ | 0 | 0 | 0 |
| 5 | 10 | 8 | $-1.5$ | 0.5 | 5 | 50 |
| 6 | 12 | 5 | $-0.5$ | 0 | 0 | 0 |
| 7 | 14 | 4 | $-0.5$ | 0 | 0 | 0 |
| 8 | 16 | 3 | $-0.5$ | 0 | 0 | 0 |
| 9 | 18 | 2 | $-0.5$ | 0 | 0 | 0 |
| 10 | 20 | 1 | $-0.5$ | 0.25 | 5 | 50 |
| 11 | 22 | 0 | 0 | 0 | 0 | 0 |
|  |  |  |  |  | 10 | =sum |

In Fig. 4a we have a stem with two vessel lengths ( 10 and 20 cm ) all starting and ending 'randomly' along the $X$ axis (it is traditional illustrated as equally spaced which is not the same as randomly spaced). $N=$ the number of paint filled vessels versus distance, $x$, as plotted in Fig. 4b. The first and second differences are calculated as shown in the table then $x$ times the second difference is computed. Each difference is the number to the left of the difference minus the number below, e.g., at $x=10$ we have $(8-5) /(10-12)=-1.5$ for the first difference and $[-1.5$ to $(-0.5)] /(10-12)=0.5$ for the second difference. The second difference is the number of vessels of the size class divided by $\Delta x$; so for the short vessels in Fig. 4 this is $10 / 2=5$. The sum of the $x$ (second difference) column (10) is used to calculate the percentage of vessels in each size class. The table can be quickly computed using Excel and is shown here to prove that the correct answer is achieved in the DD algorithm, i.e., $50 \%$ of the vessels are 10 cm long and $50 \%$ are 20 cm long (Fig. 4c)
the next larger positive value (the so-called Zimmermann correction algorithm). These unreasonable values were assumed to be counting errors and different 'corrections' to fix the problem have been proposed by Zimmermann and Jeje (1981) and by Ewers and Fisher (1989).

Ewers and Fisher (1989) argued that it is unreasonable to assume that vessel lengths are in just a few discrete size classes and hence they suggested subtracting the negative size classes from the larger positive classes but then dividing the positive value over missing size classes in between (See Fig. 5). They also thought that many of the negative size classes resulting from the DD algorithm might result just from counting errors. It is easy to show that minor adjustments in the counts (applied to real data sets) have large impact on histogram bars. The use of rubber has overcome this difficulty because 'counting errors' cannot be attributed to poor filling of vessels with pigment.


Fig. 5 A hypothetical vessel length distribution was created (a) to compute paint-filled vessel counts as in Fig. 4. The DD algorithm was then applied to the table of values to reconstruct the vessel histogram (b). The difference between (a) and (Fig. 4) is the large number of vessel size classes. When the size classes are adjacent, the algorithm slightly overestimates frequency of short vessels and underestimates frequency of long vessels. c Another hypothetical vessel length distribution like Left (a) was used plus a distinct (non-random) size

Tyree (1993) advanced the analysis by looking at how the DD algorithm reproduces known vessel length distributions. He ran models on hypothetical shoots with known vessel length distributions and has pointed out that the DD algorithm commonly overestimates vessel probabilities in the smaller size classes and underestimates probabilities in the larger size classes (Fig. 5) and that neither the Zimmermann nor the Ewers-Fisher corrections work perfectly. But Tyree (1993) did not consider the impact of 'clustering' of vessel ends that is to be expected in truly random vessel-end distributions (see caption of Fig. 5 for details). Additionally, more might be involved than just counting errors. Perfused paint particles might pile up closer to the injection surface after some paint has traveled to the vesselend, leaving gaps without paint. This would make vessels appear and disappear (as visualized by filling with pigment) and could contribute to the "impossible negative probabilities".

## Impact of clustering on the DD-algorithm

The examples of 'random' vessel ends used by Zimmermann (1983) are not really random but rather are equally spaced. Integer counts of truly random events are clustered

class ( 26 cm ) beyond the smooth distribution. The DD algorithm resolves this size class quite well in a. However, if the non-random size class is too close to the random groups (d) then the DD algorithm 'steals' some size classes from the random groups and adds them to the non-random group. $\mathrm{DD}+\mathrm{Z}=$ application of the Zimmermann correction for non-random distributions and $\mathrm{DD}+\mathrm{E} \& \mathrm{~F}=$ the application of the Ewers and Fisher correction for non-random distributions
by the very nature of random events and this point has been overlooked for 30 years. If you used a random number generator program to compute 10 decimal numbers from $x=0.000$ to $0.999 \ldots$ those numbers would be randomly spaced in the real number domain; if they were equally spaced then they would not be random. The unreasonable negative values and correction algorithms explained above are primarily the result of random fluctuations which are exacerbated by counting errors but not caused solely by counting error. In other words, even if all counts were precise the randomness of integer counts would still lead to negative values in the PDF plots. In this regard, the Skene and Balodis (1968) approach is less error prone (more robust), because they filtered out the randomness through curve fitting before applying the equivalent of a DDalgorithm to decimal values rather than to integer values.

The decay of radioactive nuclei in the time domain is another example of random events. A Geiger counter makes this clustering obvious when the rates are slow enough to discern time delays; similarly, the clustering of random vessel ends will be more like the clustering of dots in between the brackets: (... . .. . ...). The DD-algorithm is very sensitive to random clustering when applied to integer values and we can use a statistical estimate of clustering to
demonstrate it. The number of radioactive decays $(N)$ in a time period (say 1 min ) has a standard deviation of $\mathrm{SD}=N^{1 / 2}$. Hence if $N=10^{4}$ or 100 the $\mathrm{SD}=100$ or 10 , respectively. By analogy we can say a vessel count of $10^{4}$ will have a $\mathrm{SD}=1 \%$ of $N=10^{4}$ and $10 \% N=100$. Dr. Shabti Cohen (personal communication) reasoned that the random ending of vessels should follow the statistics of radioactive decay, which is exponential. Figure 6a shows a smooth exponential decay process which is linearized by a natural $\log$ plot $(\operatorname{Ln}(N)$ versus distance, $x$, which yields a straight line). $N$ can be either the total number of vessels in a stem or the number per unit area. In order to demonstrate the enormous impact of even small random errors we give an example with very small random errors $=0.5$ times the SD.

In Fig. 6a we have plotted 'random values' that are alternately $\pm 0.5$ times the SD above and below the exponential line. We used these randomized values in a DD algorithm and one example of the output is shown in Fig. 6b. Quite large deviations from the actual probability distribution function ( $\mathrm{PDF}=$ gray bars) are obviously introduced by the DD algorithm (white and black bars); hence this algorithm does not work well when statistical fluctuations cause clustering of vessel-ends of the magnitude that would be expected with normal stochastic processes. The reader can repeat the computation in Fig. 6b using alternate errors of just one SD, and it will be seen that negative values result.

The Cohen method overcomes the impact of clustering

At about the same time as the adoption of the rubberinjection method, Cohen et al. (2003) have overcome the above limitations of the DD algorithm by using a different computational algorithm that leads to a well-defined distribution function to which statistics can be applied. Cohen et al. (2003) still assume vessels end randomly in space, but they point out that an exponential extinction function is the logical consequence of this random end assumption, which is a point which also escaped the notice of Skene and Balodis (1968). An analogy is radioactive decay. A radioactive molecule comes to an end at an instant in time when the nucleus disintegrates. The random end of a radioactive substance in the time domain is analogous to the random end of a vessel in the distance domain. Hence the number of vessels remaining versus distance should decrease exponentially with distance in the same way as the number of radioactive molecules remaining at a time should decrease exponentially with time. Cohen et al. (2003) and Sperry et al. (2005) experimentally confirmed this exponential decay for the air-injection and rubberinjection methods, respectively, which implies an equation of the form


Fig. 6 a Solid line is a smooth exponential decay plotted in a natural $\log$ transform. The scatted points above and below the line are $\pm 0.5$ times the standard deviation expected for random events where $\mathrm{SD}=N^{1 / 2}$. The regression values show slope and intercept for the two cases. Note that the $R^{2}$ values are near 0.995 . b Even quite small random errors have a large impact on computed PDFs. The gray bars are the actual PDF values. DD Z means PDF computed with the DD algorithm and the Zimmermann correction and DD E\&F means DD with the Ewers and Fisher correction
$N=N_{\mathrm{o}} \exp \left(\lambda_{\mathrm{v}} x\right)$,
where $N_{\mathrm{o}}=$ the number of vessels (or number per unit area) filled at $x=0$ and $N$ is the number at $x>0$, and $\lambda_{\mathrm{v}}$ is a fitting coefficient (a negative quantity for an exponential decay); hence
$\ln (N)=\ln \left(N_{0}\right)+\lambda_{v} x$
From this Cohen et al. (2003) and Sperry et al. (2005) derived the PDF shown in Eq. (3) below by using the differential equation embodied in the DD-algorithm. Equation (3) is equal to the second derivative of Eq. (2) times $x$. Subsequently Cohen (personal communication) identified his PDF to be mathematically equivalent to a gamma distribution with parameters $(2, \lambda)$, where 2 is the shape parameter and $\lambda$ is the inverse of a scale parameter (usually symbolized as $\theta$ in gamma distribution PDFs):
$P_{x}=x \lambda_{\mathrm{v}}^{2} \exp \left(\lambda_{\mathrm{v}} x\right)$
In Eq. (3) $P_{x}$ is the probability of vessels of length $x . P_{x}$ has units of inverse distance; the distance unit is there so that
the area under the PDF curve equals a dimensionless probability of 1 (or $100 \%$ ). When Eq. (3) is represented as a bar histogram, then each bar is a size class and the height is the probability without units so the sum of the bars should equal 1 or $100 \%$. Gamma functions have welldefined statistical properties. For a gamma PDF with shape factor $=2$, the mode $=-1 / \lambda_{\mathrm{v}}$ ( $=$ the most common length ) and the mean vessel length, $\bar{L}_{\mathrm{v}}=-2 / \lambda_{\mathrm{v}}$. Note that the mean refers to the mean of all the vessels in a cross section at the point of infusion. It is important to note that this mean vessel length is not equal to the mean of all the vessels in a volume of stem, which would be an acceptable but different definition of 'population' mean.

Equation (3) was derived from the mathematical definition of the DD algorithm, but instead of computing $P_{x}$ from discrete, adjacent values of $N$ it is computed continuously from a best-fit linear regression of the log-transformed exponential decay, thus eliminating the 'counting errors' and 'unreasonable' zero and negative probabilities that result from the discrete DD algorithm which might not be the result of counter errors but rather driven by the stochastic of random clustering of vessel ends. If the literature had followed the curve-fitting approach of Skene and Balodis (1968), the analysis of vessel length distributions (PDFs) would have advanced much more rapidly as explained below but first we will continue with the history of analysis because this will explain some deviations from log-linear fits that addresses the need for more studies in the future.

Cai et al. (2010) used the Cohen method to address the question: 'Are wide vessels (large-diameter vessels) also long vessels?' When examining mean vessel lengths ( $\bar{L}_{\mathrm{v}}$ ) between species, $\bar{L}_{\mathrm{v}}$ seems to increase with mean vessel diameter $\left(\bar{D}_{\mathrm{v}}\right)$. Readers are directed to Jacobsen et al. (2012) for an excellent meta-analysis of vessel lengths. Ewers et al. (1990) showed that the maximum vessel diameter correlated linearly with maximum vessel length between species of woody vines and shrubs $\left(R^{2}=0.62\right.$ $p=0.001$ ). Hacke et al. (2006) have summarized betweenspecies values ( 28 species) of $\bar{L}_{\mathrm{v}}$ and $\bar{D}_{\mathrm{v}}$, and showed that a plot of $\log \left(\bar{L}_{v}\right)$ vs $\log \left(\bar{D}_{\mathrm{v}}\right)$ has a slope of 1.48 and $R^{2}=0.63$. Cai et al. (2010) use the symbols $L_{\mathrm{c}}$ and $D_{\mathrm{c}}$ to indicate the vessel length and vessel diameter in a bin size class, respectively.

An examination of a few species indicates a dependence of $L_{\mathrm{c}}$ on $D_{\mathrm{c}}$ but not enough examples are published to know if this dependence follows a specific function, e.g., linear or exponential. Two cottonwood clonal hybrids plus an aspen species were studied by Cai et al. 2010. In one the $L_{\mathrm{c}}$ versus $D_{\text {c }}$ was clearly linear, another was clearly exponential, and the third was curvilinear, but the $R^{2}$ was better for the linear than the exponential model (see Fig. 3 in Cai et al.


Fig. 7 These log-linear plots are examples of number of vessels per unit area, $N$, versus distance from injection surface in 4 diameter size classes. Points are means and SD of five branches. The $y$-axis is a natural $\log$ transform with linear regressions shown. Average vessel length of each diameter size class equals $-2 /$ slope
2010). The method of obtaining these curves requires a slight modification of the Cohen method. Instead of counting rubber-filled vessels, the diameter of every rub-ber-filled vessel in each section is measured. Then the vessels are divided into bin size classes and plot the $N$ in each bin size class versus $x$ for analysis by the Cohen method. This is repeated for each size class to yield the graphs shown in Fig. 7 (re-plotted from Cai et al. 2010).

## Can we divine the truth?

Can we divine the truth about vessel length PDFs using the Cohen method? The Cohen method assumes random vessel ends and through mathematical analysis this assumption leads to a prediction of log-linear plots as in Fig. 6. Furthermore, the mathematical consequence of log-linear plots leads to the statistical prediction of a specific class of PDFs (class 2 gamma distributions). But how do we confirm that vessel length PDFs are closer to a class 2 gamma distribution than some other variant function?

The way to advance studies is to return to the more general approach taken by Skene and Balodis (1968), which unfortunately was not explained clearly in the original text. We interpret their approach as follows: (1) Infuse stem segments with a colorant that travels the full length of cut open vessels. (2) Count the number ( $N$ ) of open (colored) vessels versus distance from the infusion point. (3) Obtain a smoothed curve fit to these data, i.e., a

Fig. 8 a A theoretical plot of natural log of vessel counts versus distance for three cases that are not class 2 gamma functions to illustrate the extent of non-linearity. $\mathbf{b}$ The residuals of $\mathbf{a}$. See text for details. $\mathbf{c}$ and $\mathbf{d}, \mathbf{e}$ and $\mathbf{f}$, and $\mathbf{g}$ and $\mathbf{h}$ are similar to $\mathbf{a}$ and $\mathbf{b}$ except we plot real values and show real residuals for 3 Populus species or hybrids. See text for details

function $N=f(x)$. (4) Perform a DD computation based on the smoothed function from which a vessel-length distribution curve (PDF) can be calculated. Cohen et al. (2003) took an approach that was close to but not exactly like the above four steps, because they assumed random vessel ending that mathematically resulted in a theoretical function that was exponential for $N=N_{\mathrm{o}} \exp (\lambda x)$. This assumption leads specifically to a class 2 gamma distribution for the PDF after DD computation ( $x \frac{\mathrm{~d}^{2} N}{\mathrm{~d} x^{2}}$ which yields Eq. 3). Hence finding a more correct distribution requires doing measurements to see how much the actual function of $N=f(x)$ differs from the log-transformed
function in Eq. (2), because divergence from a log-linear function implies a different PDF for real stems.

It is also possible to take a theoretical approach to how much other simple distribution functions would affect the shape of the log-transformed plots. This will provide some guidance on whether you would detect other PDF values by looking at the deviations from linearity in log-transformed data. This theoretical exercise is done in Fig. 8 a and b , for three cases: (1) Assuming all vessels are the same length $(10 \mathrm{~cm})$, but they begin and end in random locations along the stem axis, which we call a single distribution. (2) A flat distribution where there are 10 groups of vessels where
$10 \%$ of the vessels in each group are $1,2,3 \ldots 10 \mathrm{~cm}$ long, but begin and end in random locations along the stem axis, and (3) an approximately normal distribution of 11 groups with the mean length being 6 cm , the shortest 1 cm and the longest group 11 cm . The log-transformed theoretical data are shown in Fig. 8a. None of the regressions are particularly linear, but the $R^{2}$ values are all $>0.9$. All of the curves show convex residuals (=the deviation from the regression line and the actual value) as shown in Fig. 8b. These residual deviations would be easily detectable in an experimental data set, but so far the residuals seen in real data sets are smaller and concave rather than convex.

Figure 8c, e, and g shows the deviations from linearity from the data sets published in Cai and Tyree (2010) for three Populus clones/species; and the residuals are all smaller and concave rather than convex. From this we conclude that real vessel length distributions are not single, flat or normal. Cai and Tyree (2010) provided data in support of the notion that large-diameter vessels are longer than small-diameter vessels. We also know that small-diameter vessels are more numerous than largediameter vessels, so the consequence of this would be concave residuals. In Fig. 9a we show the log-transformed data from Fig. 8e fitted with a second-order polynomial which increases the $R^{2}$ from 0.993 to 0.9993 . Another measure of the closeness of fit is the root-meansquare error ( $E_{\mathrm{rms}}$ ), which is mathematically similar to the standard deviation of the data points around the bestfit line. The log-linear fit had an $E_{\text {rms }}=0.1077$ vs 0.03586 for the polynomial fit, which means that the polynomial fit is about three times more precise than the linear fit. We have used the two regressions to compute $N$ versus $x$ at 0.2 cm intervals from 0 to 5 cm , then used the DD-algorithm on the data set to compute the PDF. The solid line in Fig. 9b is the PDF for the polynomial fit and the dashed line is for the log-linear fit, which is a class 2 gamma distribution (with shape factor $=2$ ). We suspect that the solid-line PDF is more correct than the dashed-line PDF and the main difference is that there are more short vessels and fewer long vessels.

Future research might focus on obtaining log linear regressions of vessel counts starting at higher counts than normal, e.g., $N=10,000$ at $x=0$ over a larger cross section of wood area, $A$. Higher counts will reduce the standard deviation on the values of $n=N / A$ (vessel count per unit area) because if vessels end randomly in the distance domain then the standard deviation will be equal to $\sqrt{N} / A$. Reducing the standard deviation will increase the precision of $\ln (n)$ and hence the precision of the residuals, and will allow us to compute vessel-length distributions with more precision. Further insights can be gained by use of cinematographic technique (Zimmermann 1971, 1978)


Fig. 9 a Is a re-plot of Fig. 8e with a second-order polynomial fit. b The solid line shows the computed vessel length distribution based on the more accurate polynomial fit. The dashed line shows the class 2 gamma distribution computed from the linear fit. There are some vessels longer than 5 cm , but their probability distributions cannot be calculated beyond the range of measured data
or the newer high-resolution X-ray imaging techniques (Brodersen et al. 2011).

## Conclusions

Accurate vessel length determination requires the infusion of stem or root sections with a pigmented substance that will travel through many perforation plants of vessel elements but not pass through the nano-scale pores of pit membranes. Today the substance of choice is silicone rubber compound impregnated with dye that is insoluble in water but capable of being held in suspension in un-polymerized silicone rubber. Although the DD algorithm has been frequently used in the past to compute vessel-length distribution, the DD algorithm has been revealed in this review to be extremely sensitive to the clustering of vessel ends, which is the statistical consequence of random vessel ends. The Cohen method of analysis is recommended to turn counts of rubber-filled vessels into a vessel-length
distribution (PDF). The Cohen-method (Cohen et al. 2003) assumes a class 2 gamma distribution function which is mathematically consistent with a log-linear plot of count versus distance. Real plots are not exactly log-linear, which suggests a slight deviation from a class 2 gamma PDF. This deviation could be caused by vessel length being a function of vessel diameter as proposed by Cai et al. (2010), but may be due to other causes too. Researchers interested in figuring out the 'real' vessel length PDF may want to return to the statistical approaches of Skene and Balodis (1968), which place no restriction on the real PDF as shown in Fig. 9.

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