Ready for Screening: Fast Assessable Hydraulic and Anatomical Proxies for Vulnerability to Cavitation of Young Conifer Sapwood

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Abstract: Research Highlights: novel fast and easily assessable proxies for vulnerability to cavitation of conifer sapwood are proposed that allow reliable estimation at the species level. Background and Objectives: global warming calls for fast and easily applicable methods to measure hydraulic vulnerability in conifers since they are one of the most sensitive plant groups regarding drought stress. Classical methods to determine \( P_{25} \), \( P_{50} \) and \( P_{88} \), i.e., the water potentials resulting in 12, 50 and 88\% conductivity loss, respectively, are labour intensive, prone to errors and/or restricted to special facilities. Vulnerability proxies were established based on empirical relationships between hydraulic traits, basic density and sapwood anatomy. Materials and Methods: reference values for hydraulic traits were obtained by means of the air injection method on six conifer species. Datasets for potential \( P_{50} \) proxies comprised relative water loss (RWL), basic density, saturated water content as well as anatomical traits such as double wall thickness, tracheid lumen diameter and wall/lumen ratio. Results: our novel proxy \( P_{25W} \), defined as 25\% RWL induced by air injection, was the most reliable estimate for \( P_{50} (r = 0.95) \) and \( P_{88} (r = 0.96) \). Basic wood density \((r = -0.92)\), tangential lumen diameters in earlywood \((r = 0.88)\), wall/lumen ratios measured in the tangential direction \((r = -0.86)\) and the number of radial cell files/mm circumference \((CF/mm, r = -0.85)\) were also strongly related to \( P_{50} \). Moreover, \( CF/mm \) was a very good predictor for \( P_{12} (r = -0.93) \). Conclusions: the proxy \( P_{25W} \) is regarded a strong phenotyping tool for screening conifer species for vulnerability to cavitation assuming that the relationship between RWL and conductivity loss is robust in conifer sapwood. We also see a high potential for the fast and easily applicable proxy \( CF/mm \) as a screening tool for drought sensitivity and for application in dendroecological studies that investigate forest dieback.

Keywords: conifers; biodiversity; drought stress; forest dieback; hydraulic capacitance; phenotyping; structure–function relationships; vulnerability to cavitation; wood anatomy

1. Introduction

Current models predict widespread forest mortality due to global warming [1–3]. Conifers are amongst the most endangered plant groups regarding tree mortality [4,5] and forest dieback [6] induced by drought and heat waves. As a first step in drought response, trees close stomata in order to limit both water loss and further decrease in (secondary) xylem water potential. Water loss via needles proceeds thereafter at a much lower rate, as the water potential becomes more negative, the water columns in the tracheids can break depending on the cuticular resistance, which itself is influenced by temperature [7]. When the water potential becomes more negative, the water columns in the tracheids can break (cavitation) more easily followed by further development and spread of embolisms. As the number of emboli increases, conductivity loss in the (secondary) xylem also increases [8,9]. This dynamic process can be simulated by vulnerability curves, where the percentage loss of conductivity is plotted against the water potential. In conifers, \( P_{50} \), i.e., the water potential resulting in 50\% conductivity loss derived from the vulnerability curves, is regarded as the ”point of no return” for recovery from drought, as it represents the minimum...
recoverable water potential in many species [9]; exceptions e.g., [10]. The hydraulic safety margin of a species is calculated from the difference between the minimum water potential measured in the field and \( P_{50} \) [9,11–13]. Information on \( P_{50} \) can thus be helpful to screen for more drought susceptible conifer species or provenances. The correct determination of \( P_{50} \) by means of classical flow experiments is, however, labour intensive and difficult because measurement errors can occur during repeated flow experiments; resin can clog the conducting system or native embolism might obscure the results [14,15]. In our study we aimed at developing fast and easily applicable alternatives for estimating the species’ specific hydraulic vulnerability of young sapwood.

In conifers, capacitive water loss [16,17] is strongly related to hydraulic conductivity loss due to their quite homogenous and “simple” wood structure [18]. The \( P_{50} \) of sapwood corresponds to 25.18% of relative water loss (RWL) across conifer species (“conifer-curve”) [19]. A hydraulic alternative to \( P_{50} \) could thus be the water potential resulting in ~25% relative water loss (\( P_{25W} \)), whereby RWL can be obtained by simple gravimetric measurements. Anatomical proxies for \( P_{50} \) are based on the assumption that the resistance against implosion, which should depend on the wall (\( t \)) to lumen (\( b \)) ratio (\( t/b \)), is strongly linked to vulnerability to cavitation [20,21]. In other words, sapwood that can withstand lower water potentials before cavitation occurs must be constructed more safely and should thus have a higher \( t/b \) or \((t/b)^2\), which is termed “conduit wall reinforcement”. The relationship between \( t/b \) and \( P_{50} \) is tighter across conifer species than the relationships between pit anatomical traits and \( P_{50} \) [22]. Several approaches to estimate proxies for \( P_{50} \) based on tracheid dimensions can be found in the literature; measurements are either performed in the initially formed tracheid rows [23], in the entire earlywood [22,24], or in several radial files across the whole annual ring, where \((t/b)^2\) is thereafter assessed on tracheids that are in the range of defined hydraulic diameters e.g., [20,25–27]. Refs. [24,27] detangled tangential and radial lumen diameters when calculating \((t/b)^2\) as a proxy for \( P_{50} \), because of the much tighter relationships between tangential lumen dimensions and \( P_{50} \). Anatomical proxies for \( P_{50} \) offer the advantage that samples do not need to be fresh and that hydraulic vulnerability can be determined retrospectively for selected annual rings. The approach of relating drought response of trees to “constitutive wood anatomy” is based on works by [27,28] who found that the wood formed (often several years) before drought stress impacts the sensitivity to drought in sapwood of *Picea abies* and different *Larix* species. Drought induced dieback [27] or growth decreases [28] are more strongly related to \((t/b)^2\) measured in the tangential rather than in the radial direction. Wood density is a very good proxy for hydraulic vulnerability in conifers [20,29] and is easy to measure; however, when the intention is a retrospective analysis at the annual ring level, techniques such as X-ray or high-frequency densitometry need to be applied [30,31]. Since such equipment is not readily available, the use of reliable anatomical proxies could offer a viable alternative in dendroecological research.

The aim of this study is to establish fast, easily applicable and reliable methods to estimate the hydraulic vulnerability of sapwood. We tested both hydraulic (\( P_{25W} \)) and anatomical proxies such as lumen diameters, \((t/b)^2\) and the number of radial cell files/mm circumference, as well as basic wood density, for their predictive quality of \( P_{12} \), \( P_{50} \) and \( P_{88} \), i.e., the water potentials resulting in 12, 50 and 88% conductivity loss, respectively. We included \( P_{12} \) and \( P_{88} \) in our analyses because recent studies show that conductivity losses higher than 12% occur after stomata are already closed [8,9], but there are also hints that some conifer species are able to survive high conductivity losses [10]. Regarding anatomical traits, our intention was to find proxies for hydraulic vulnerability of wood that can be applied in dendroecological research in order to relate them to the drought stress response of trees.
2. Materials and Methods

2.1. Plant Material and Harvest

Juvenile stems and branches of six different conifer species, comprising Abies nordmanniana (STEV.) SPACH, Larix decidua MILL., Picea abies (L.) KARST., Pinus nigra ssp. nigricans HOST. (“Austrian Pine”), Pseudotsuga menziesii (MIRBEL) FRANCO and Taxodium distichum (L.) RICH., were investigated (Table 1). All sampled trees were grown in botanical gardens near BOKU University, Vienna, Austria, with transport times to the laboratory of less than 30 min. Branches (0.5–1.5 m) or whole saplings (<1 m) were harvested early in the morning and put in black plastic bags containing wet paper towels.

Table 1. Information on the age of the sampled trees (age), the amount of tree individuals investigated (trees), the sample numbers (samples) as well as the dataset numbers (dataset, single hydraulic measurements before and after repeated air injection). All specimens came from botanical gardens in Vienna, Austria, latitude 48°14′12″ N–48°14′33″ N and longitude 16°18′21″ E–16°20′15″ E.

| Species                  | Age | Trees | Organ | Samples | Dataset |
|--------------------------|-----|-------|-------|---------|---------|
| Abies nordmanniana       | 4   | 3     | stem  | 4       | 27      |
| Larix decidua            | 20  | 2     | branch| 8       | 52      |
| Picea abies              | 4   | 8     | stem  | 8       | 54      |
| Pinus nigra              | 20  | 3     | branch| 3       | 20      |
| Pseudotsuga menziesii    | 4   | 5     | stem  | 5       | 36      |
| Taxodium distichum       | 20  | 3     | branch| 6       | 36      |

2.2. Reference Values of Hydraulic Vulnerability to Cavitation (P$_{50}$, P$_{12}$, P$_{88}$)

Reference values for P$_{50}$ were obtained by the air injection method, where the application of positive pressure in a double ended pressure sleeve mimics the water potential [32,33] and eventually induces cavitation and moisture loss [18]. The air injection method is suitable for conifer branches, trunks and roots [34].

Stem segments with a length of 200 mm were debarked under water and re-saturated under low vacuum for 24 h at 4°C in filtered (0.22 µm), distilled water [35] with 0.005% Micropur (Katadyn Products, Wallisellen, Switzerland). Specimens were shortened to 130 mm and re-cut several times with razor blades, resulting in a final length of about 120 mm. Hydraulic conductivity was measured under a pressure head of 5.4 kPa with distilled and filtered water containing 0.005% Micropur. Air injection ($\Psi$) was applied in a double-ended pressure chamber (PMS Instruments, Corvallis, OR, USA) for one minute [18]. Samples were allowed to equilibrate for 30 min under water. Thereafter, the hydraulic conductivity was measured again and the percent loss of conductivity was calculated. The pressure applied in each air injection was gradually increased by steps of 0.5 or 1.0 MPa.

In a hydraulic vulnerability curve, the percent loss of conductivity (PLC) is plotted against the water potential ($\Psi$). The application of positive pressure (air injection) mimics a decrease in water potential, and, therefore, $\Psi$ is hereafter used to refer to both water potential and pressure application. Hydraulic vulnerability curves were established separately for each sample in order to calculate P$_{12}$, P$_{50}$ and P$_{88}$ values [36], corresponding to the pressure application at which 12, 50 or 88% of conductivity loss occurred. The trait $\Psi_{12}$ is termed the “air entry point” and is an estimate of the water potential at which the resistance to air entry of the pit membranes is overcome and cavitation and embolism is likely to start. Based on the latter, $\Psi_{88}$ is termed the “full embolism point”, which is the water potential close to the state when the sapwood becomes non-conductive [36]. Calculation of P$_{12}$, P$_{50}$ and P$_{88}$ values was done by means of the exponential sigmoidal Equation (1) [37].

$$PLC (%) = 100/(1 + \exp(a (\Psi - b)))$$ (1)

In Equation (1), “a” corresponds to the slope of the linear part of the function and “b” is the P$_{50}$. 
2.3. Relative Water Loss and Calculation of \( P_{25W} \)

After full saturation and before the first hydraulic flow measurement, the saturated weight (SW) of the specimen was determined on a lab balance (resolution of 0.0001 g, Mettler Toledo International Inc., Greifensee, Switzerland). The fresh weight (FW) was subsequently determined after each pressure application. In order to assess the dry weight (DW), specimens were dried for 48 h at 103 °C [29]. The relative water loss (RWL) was calculated with Equation (2).

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RWL (%) = 100 \left( 1 - \frac{(FW - DW)}{(SW - DW)} \right)
\]  

Relative water loss curves, i.e., the RWL plotted against \( \psi \) were fitted by the “curve estimation” and “non-linear regression” functions in SPSS™ 21.0. The fittings with the highest predictive quality (\( r^2 \)) and the most reliable shape were chosen, comprising quadratic, cubic and Weibull functions [19,38] in order to calculate \( P_{25W} \), defined as the pressure application, i.e., water potential, resulting in 25% of RWL.

2.4. Basic Wood Density

Basic wood density (BD) is the mass in the oven dry state divided by the sample volume in the fully saturated, never dried, green state [kg/m^3]. Directly after the first hydraulic flow measurement, dimensions in the fully saturated state were measured with a caliper (resolution of 0.1 mm). The mass in the oven dry state was assessed after specimens were dried for 48 h at 103 °C [29].

2.5. Wood Anatomy

Segments with a length of 3 cm were sawn from the specimens on which the hydraulic reference data were measured. Wood samples were softened in a mixture of water, alcohol and glycerol (1:1:1) for at least two weeks. Transverse sections with a thickness of 20 µm were produced on a sliding microtome (Jung Reichert, Vienna, Austria). Sections were stained with Methylene blue, dehydrated with alcohol and mounted in Euparal (Merck, Darmstadt, Germany). Microphotography was achieved with a Leica DM4000 M microscope equipped with a Leica DFC320 R2 digital camera and Leica IM 500 Image Manager image analyzing software (Leica, Wetzlar, Germany). Cell wall thickness and tracheid dimensions were measured by means of Image J software [39] in the first ten radial earlywood cell files [28] of the latest annual ring in four different locations around the stem. Lumen diameters (\( b \)) and double cell wall thickness (\( t \)) in the radial (\( b_r, t_r \)) and tangential (\( b_t, t_t \)) directions of 20 tracheids (Figure 1a,b) were measured. From these traits, the conduit wall reinforcements [20] in the radial ((\( t_r/b_r \)) and in the tangential ((\( t_t/b_t \)) direction were calculated. In addition, the number of radial cell files/mm circumference (CF/mm) was determined for each location. Care was taken, that in the regions investigated (a) no ray cells were present and that (b) tracheids were not cut transversally at their tips (Figure 1c,d). A suitable approach was to select a tangential row of 5–10 radial tracheid files and measure the distance from the middle lamellae of the first to the middle lamellae of the last tracheid in this row. The number of radial tracheid files was then related to 1 mm of circumference.
Figure 1. Description of anatomical traits; (a) one annual ring of *Picea abies* stem wood; (b) anatomical traits measured in earlywood; (c) *Abies nordmanniana* stem wood; (d) *Taxodium distichum* branch wood; the pink bars in (c,d) indicate regions of interest for counting of radial cell files in earlywood; $b_r$ radial tracheid lumen diameter; $b_t$ tangential tracheid lumen diameter; $t_r$ radial tracheid double cell wall thickness, $t_t$ tangential tracheid double cell wall thickness.

2.6. Sample Numbers, Data Processing and Statistical Analyses

Hydraulic and anatomical traits (Table 2) of six different conifer species with 3–8 replicates/species were investigated (Table 1). Except for *P. nigra*, raw datasets for hydraulic traits ($\Psi$, PLC, RWL) were available [18]. However, for the present work, vulnerability curves and relative water loss curves were not calculated for pooled datasets but separately for each sample. Statistical analysis was carried out with SPSS$^\text{TM}$ 21.0. Normal distribution was tested with the Kolmogorov–Smirnov test. Species mean values of all traits were normally distributed, whereas some sample specific trait datasets were not (lumen diameters and conduit wall reinforcements). In order to meet the requirement for normality, data of these traits were transformed into normal scores by calculating their logarithmic values.
Relationships between all traits were tested by Pearson correlation and selected traits by linear regressions. Relationships were accepted as significant if the \( p \)-value was <0.05.

### Table 2. List of traits and their abbreviations.

| Abbreviation | Trait | Unit |
|--------------|-------|------|
| \( P_{12} \) | Water potential resulting in 12% conductivity loss | MPa |
| \( P_{50} \) | Water potential resulting in 50% conductivity loss | MPa |
| \( P_{88} \) | Water potential resulting in 88% conductivity loss | MPa |
| \( P_{25W} \) | Water potential resulting in 25% relative water loss | MPa |
| BD | Basic wood density | kg m\(^{-3}\) |
| \( b_t \) | Radial lumen diameter of earlywood tracheids | \( \mu m \) |
| \( t_r \) | Thickness of the radial double cell wall of earlywood tracheids | \( \mu m \) |
| \( (t_r/b_t)^2 \) | Radial conduit wall reinforcement | dimensionless |
| \( b_t \) | Tangential lumen diameter of earlywood tracheids | \( \mu m \) |
| \( t_t \) | Thickness of the tangential double cell wall of earlywood tracheids | \( \mu m \) |
| \( (t_t/b_t)^2 \) | Tangential conduit wall reinforcement | dimensionless |
| \( b \) | Mean lumen diameter \((b_t + b_t)/2\) | \( \mu m \) |
| \( t \) | Mean double cell wall thickness \((t_r + t_r)/2\) | \( \mu m \) |
| \( (t_tb_t)^2 \) | Mean conduit wall reinforcement \((({b_t}/b_t)^2 + (t_t/b_t)^2)/2\) | Dimensionless |
| CF/mm | Number of radial cell files per tangential distance of 1 mm | n/mm |

### 3. Results

#### 3.1. Range of Hydraulic Vulnerabilities

Conifer samples varied widely in their hydraulic vulnerabilities \( P_{12}, P_{50}, P_{88} \), whereby the lowest values were found in \textit{A. nordmanniana} stems, intermediate in \textit{L. decidua} and \textit{P. nigra} branches and the highest in \textit{T. distichum} branches (Table 3).

| Species | \( P_{50} \) [MPa] | \( P_{12} \) [MPa] | \( P_{88} \) [MPa] | \( P_{25W} \) [MPa] |
|---------|----------------|----------------|----------------|----------------|
| \textit{Abies nordmanniana} | \(-8.07 \pm 0.78\) | \(-4.11 \pm 1.62\) | \(-12.24 \pm 0.62\) | \(-8.58 \pm 0.78\) |
| \textit{Larix decidua} | \(-4.42 \pm 0.31\) | \(-2.70 \pm 0.26\) | \(-6.14 \pm 0.55\) | \(-5.09 \pm 0.37\) |
| \textit{Picea abies} | \(-6.26 \pm 0.46\) | \(-3.70 \pm 0.50\) | \(-8.82 \pm 0.73\) | \(-6.40 \pm 0.62\) |
| \textit{Pinus nigra} | \(-4.33 \pm 0.12\) | \(-3.06 \pm 0.35\) | \(-5.60 \pm 0.22\) | \(-2.97 \pm 0.22\) |
| \textit{Pseudotsuga menziesii} | \(-5.14 \pm 0.84\) | \(-3.47 \pm 0.53\) | \(-6.82 \pm 1.43\) | \(-5.28 \pm 0.83\) |
| \textit{Taxodium distichum} | \(-2.30 \pm 0.31\) | \(-0.71 \pm 0.58\) | \(-4.19 \pm 0.55\) | \(-2.28 \pm 0.44\) |

#### 3.2. Relationships between Hydraulic Traits

Species-specific relative water loss curves (Figure 2a) showed the same species ranking as hydraulic vulnerability curves (Figure 2b): \textit{T. distichum} had both the steepest decline in relative water loss (RWL) as well as in hydraulic conductivity loss (PLC), whereas \textit{A. nordmanniana} had the lowest decline in RWL and PLC with decreasing \( \Psi \). Mean \( P_{25W} \), i.e., the \( \Psi \) resulting in 25% relative water loss, was strongly related to mean \( P_{50} \) across species \( r = 0.95 \), Figure 3). By excluding, \textit{P. nigra}, which had either a higher \( P_{25W} \) (underestimated) or a lower \( P_{50} \) (overestimated) than expected, the relationship would have become even tighter \( r = 0.99, p \leq 0.001 \). Tight relationships were also found between mean \( P_{25W} \) and \( P_{88} \) \( r = 0.96 \), since \( P_{88} \) was strongly related to \( P_{50} \) \( r = 0.98 \) across species (Table 4).
or a lower $P_{50}$ (overestimated) than expected, the relationship would have become even tighter ($r = 0.99$, $p \leq 0.001$). Tight relationships were also found between mean $P_{25W}$ and $P_{88}$ ($r = 0.96$), since $P_{88}$ was strongly related to $P_{50}$ ($r = 0.98$) across species (Table 4).

Figure 2. Empirical hydraulic traits; (a) relative water loss (RWL) and (b) percent loss of hydraulic conductivity (PLC) plotted against the negative of the air pressure applied in a pressure collar (water potential) for six different conifer species. Error bars indicate one standard error. Curves for mean values were fitted by a Weibull equation [38].

Figure 3. Relationships between $P_{50}$ (water potential resulting in 50% conductivity loss) and $P_{25W}$ (water potential resulting in 25% relative water loss) of six different conifer species. Small dots indicate single tree or branch samples; big dots species mean values. Hatched lines are linear regression lines for single tree or branch samples, solid lines are linear regression lines for species mean values.
Table 4. Correlation matrix for hydraulic and wood anatomical traits. Numbers in the upper right are the Pearson correlation coefficients of species mean values (n = 6), numbers in the lower left of the sample values (vulnerability curves, n = 34). The significance level is indicated with * if p < 0.05, ** if p ≤ 0.01 and *** if p ≤ 0.001.

|       | $P_{50}$ | $P_{12}$ | $P_{88}$ | $P_{25W}$ | BD     | $b_r$  | $t_r$  | $(t_r/b_r)^2$ | $b_t$  | $t_t$  | $(t_t/b_t)^2$ | $b$    | $t$    | $(t/b)^2$ | CF/mm |
|-------|----------|----------|----------|-----------|--------|--------|--------|---------------|--------|--------|---------------|--------|--------|------------|--------|
| $P_{50}$ | 0.91 **  | 0.98 *** | 0.95 **  | −0.92 *   | 0.53   | −0.54  | −0.74  | 0.88 *        | −0.44  | −0.86 * | −0.71        | −0.50  | −0.81 * | −0.85 *    | −0.85 * |
| $P_{12}$ | 0.86 *** | 0.80     | 0.81 *   | −0.78     | 0.66   | −0.21  | −0.57  | 1.00 ***      | −0.05  | −0.58  | −0.13        | −0.58  | 0.84 *  | −0.13      | −0.93 ** |
| $P_{88}$ | 0.95 *** | 0.68 *** | 0.96 **  | −0.92 **  | 0.44   | −0.69  | −0.79  | 0.76         | −0.62  | −0.94 ** | 0.60         | −0.66  | −0.88 * | −0.75      | −0.75  |
| $P_{25W}$ | 0.95 *** | 0.78 *** | 0.93 *** | −0.83 *   | 0.29   | −0.59  | −0.64  | 0.76         | −0.54  | −0.86 * | 0.50         | −0.57  | −0.76   | −0.78      | −0.78  |
| BD     | −0.67 ***| −0.41 *  | −0.74 ***| −0.63 *** | −0.50  | 0.50   | 0.74   | −0.77        | 0.44   | 0.83 *  | −0.65        | 0.47   | 0.80    | 0.74       | 0.74   |
| $b_r$  | 0.47 **  | 0.38 *   | 0.47 **  | 0.31      | −0.47 **| −0.25  | −0.72  | 0.68         | 0.00   | −0.38  | 0.96 *       | −0.13  | −0.60   | −0.66      | −0.66  |
| $t_r$  | −0.38 *  | −0.15    | −0.49 ** | −0.38 *   | 0.30   | −0.28  | 0.82 *  | −0.14        | 0.96 **| 0.88 *  | −0.23        | 0.99 **| 0.87 ** | 0.26       | 0.26   |
| $(t_r/b_r)^2$ | −0.51 ***| −0.32    | −0.58 ***| −0.39 **  | 0.48 ***| −0.82 ***| 0.76 ***| −0.54        | 0.66   | 0.87 *  | −0.71        | 0.75   | 0.98 ** | 0.65       | 0.65   |
| $b_t$  | 0.64 *** | 0.59 *** | 0.59 *** | 0.60 ***  | −0.48 **| 0.52 **| −0.01  | 0.33         | 0.02   | −0.53  | 0.86 *       | −0.06  | −0.55   | −0.93 **   | −0.93 **|
| $t_t$  | −0.22    | 0.07     | −0.38    | −0.27     | 0.21   | −0.08  | 0.89 ***| 0.57 ***      | 0.04   | 0.83 *  | 0.01         | 0.99 **| 0.75    | 0.09       | 0.09   |
| $(t_t/b_t)^2$ | −0.54 ***| −0.29    | −0.63 ***| −0.55 *** | 0.44 **| −0.34 *| 0.69 ***| 0.63 ***      | −0.60 ***| 0.76 ***| −0.47        | 0.87 * | 0.95    | 0.59       | 0.59   |
| $b$    | 0.61 *** | 0.52 *** | 0.59 *** | 0.49 **   | −0.54 **| 0.92 **| −0.20  | −0.72 ***     | 0.80 ***| −0.05  | −0.51 **      | −0.12  | −0.63   | −0.82 **    | −0.82 **|
| $t$    | −0.31    | −0.04    | −0.45    | −0.33     | 0.26   | −0.19  | 0.97 ***| 0.69 ***      | 0.01   | 0.97 ***| 0.75 ***      | −0.13  | 0.82 ** | 0.18       | 0.18   |
| $(t/b)^2$ | −0.59 ***| −0.35 *  | −0.67 ***| −0.52 *** | 0.53 **| −0.69 ***| 0.79 ***| 0.92 ***      | −0.52 **| 0.71 ***| 0.87 ***      | −0.71 ***| 0.77 *** | 0.64       | 0.64   |
| CF/mm  | −0.46 ** | −0.42 *  | −0.44 ** | −0.48 **  | 0.33 * | −0.46 **| −0.06  | 0.23         | −0.84 ***| −0.03  | 0.46 ***      | −0.69 ***| −0.05   | 0.38 *     | 0.38   |
3.3. Structure–Function Relationships

Basic wood density was the best proxy for $P_{50}$ ($r = -0.92$, Figure 4a) and was also strongly related to $P_{88}$ (Figure 4c) and $P_{25W}$ (Table 4). The species-specific vulnerability to cavitation increased with decreasing wood density. Mean values of radial lumen diameters were not significantly related to empirical hydraulic traits (Table 4), whereas mean tangential lumen diameters were significantly related to $P_{50}$ (Figure 4d) and $P_{12}$ (Figure 4e) but not to $P_{88}$ (Figure 4f). For $P_{12}$, the tangential tracheid lumen diameter was the best proxy ($r = 0.996$, Figure 4e); species with a higher hydraulic vulnerability had higher tangential tracheid lumen diameters in earlywood. Mean wall thickness traits were not significantly related to empirical hydraulic traits (Table 4). Mean tangential conduit wall reinforcement was significantly related to $P_{50}$ (Figure 4g) and $P_{88}$, (Figure 4i) but not to $P_{12}$ (Figure 4h). The best proxy for $P_{88}$ was the tangential conduit wall reinforcement ($r = -0.94$); the latter was also significantly related to $P_{25W}$ ($r = -0.86$) (Table 4). Vulnerability to cavitation increased with decreasing tangential conduit wall reinforcement. Species with a higher number of radial cell files/mm had a lower $P_{50}$ (Figure 4j), $P_{12}$ (Figure 4k) and $P_{88}$ (Figure 4l), whereby for the latter the relationship was not statistically significant.
Figure 4. Structure–function relationships of sapwood across conifer species. $P_{50}$, $P_{12}$ and $P_{88}$ are plotted against (a–c) basic wood density, (d–f) tangential lumen diameter of earlywood tracheids, (g–i) tangential conduit wall reinforcement of earlywood and (j–l) to the number of radial cell files/mm of six conifer species, indicated by different colors. Small dots indicate single samples (vulnerability curves), big dots species mean values. Solid lines denote significant linear relationships for species mean values and hatched lines represent linear regression lines that are not significant.
4. Discussion

4.1. The Hydraulic Capacitance Parameter $P_{25W}$ Is the Best Proxy for Vulnerability to Cavitation

The novel trait $P_{25W}$ had the highest predictive quality for $P_{50}$ ($r^2 = 0.91$) and the relationship between these parameters was linear across species. Data for *P. nigra* shifted most from the linear relationship (Figure 3). Either the $P_{25W}$ values were underestimated (too high, i.e. less negative), suggesting artificially high water loss (of oversaturated tissue) at a given water potential or the $P_{50}$ values were overestimated (too negative). Existing emboli in the sapwood can result in “shifting” of the vulnerability curve towards more negative water potentials resulting in more negative $P_{50}$ values [15,40]. If trees have already been subjected to water potentials low enough to cause embolism, refilling of these conduits is necessary to obtain standardized species-specific vulnerability curves [19]. In our study, refilling was done under partial vacuum [35], because night-time branch rehydration is often not sufficient to refill emptied tracheids [40]. We estimated $P_{50}$ at $-4.3$ MPa and $P_{25W}$ at $-3.0$ MPa. In previous works, $P_{50}$ values of $-3.8$ MPa and $-3.6$/$-3.2$ MPa were reported for young *P. nigra* stems [41] and branches [40], respectively. As mentioned above, oversaturation through refilling of wood that did not contribute to sap flow initially would result in an underestimation rather than an overestimation of $P_{50}$ [15] and can thus be excluded for our *P. nigra* samples. However, repeated air injection might lead to artificial outflow of resin and emptied canals might refill with water during the repeated flow measurements, resulting in an overestimation of the hydraulic conductivity at a given $\Psi$ and thus an underestimation of the conductivity loss (PLC). Such a “cut open resin canal effect” occurring at later dehydration stages would eventually result in an overestimation (more negative values) of $P_{50}$. *P. nigra* wood is known for its big resin canals and its high amount of resin produced (Figure 5). Because of the latter, *P. nigra* has been commercially used and very likely been selected for resin production [42]. Axial resin canals in *Pinus* species can reach maximum lengths of up to 1 m and mean lengths up to 0.5 m reviewed in [43], thus, most of the resin canals were probably much longer than the sample length used for the flow experiments (120 mm). If some resin was already replaced by water from cut open canals during the re-saturation process under partial vacuum, an artificial oversaturation with water would result in an underestimation (higher values) of $P_{25W}$ (Figure 3) and, if water flow was affected as well, also of $P_{50}$. The construction of hydraulic vulnerability curves of resinous conifer species remains challenging and the relationship between $P_{25W}$ and $P_{50}$ across conifer species might be useful for finding the most reliable method to estimate $P_{50}$.

Figure 5. Branch wood of *Pinus nigra* stained with astablue/safranin.
4.2. Tangentially Measured Anatomical Traits Have a Higher Predictive Quality for Hydraulic Failure

Basic wood density was the best proxy for $P_{50}$ as shown in earlier studies [20,29,44]. Wood density is, however, not that easily determined at the tree ring level, whereby techniques such as X-ray micro-density or high-frequency densitometry must be applied [24,30,31]. Differences in wood density result from changes in lumen diameter and cell wall thickness. The strongest anatomical predictive trait for $P_{50}$ was the tangential lumen diameter of earlywood ($r^2 = 0.77$). The relationship of $P_{12}$ with the latter was even stronger ($r^2 = 0.99$). In a previous work, [27] showed that top dieback in Norway spruce was more strongly related to the tangential rather than radial tracheid lumen diameters and that $(t/b)^2$ calculated from tangential lumen diameters had a higher predictive quality for $P_{50}$ than when calculated from the mean or the radial lumen diameters. Our results for the relationship of $(t/b)^2$ traits with $P_{50}$ among conifer species are in agreement with the latter. More recently, [45] showed that for Norway spruce at breast height, the tangential lumen diameters increase more with tree height than the radial diameters; as a consequence, the number of radial tracheid files/mm circumference is negatively related to tree height. Accordingly, lower hydraulic safety is found with higher distance from the treetop [44]. Tip-to-base xylem conduit widening of vessel and tracheid diameters has been reported for many species [46,47] and detangling radial and tangential lumen diameters could provide further insights in within-tree differences in hydraulic vulnerability.

Tangential lumen diameters are less influenced by annual differences in climate, because the rate of periclinal (radial: inside and outside) division is much higher than that of anticlinal (circumference: side) division [48]. The number of radial tracheid files/mm circumference (CF/mm) in earlywood depends on the tangential lumen diameters rather than on the cell wall thickness. As the tangential lumen diameters of earlywood tracheids and the CF/mm showed a similar or even stronger predictive quality for $P_{50}$ than the best $(t/b)^2$ trait (Table 4), we suggest that it is not necessary to calculate conduit wall reinforcement traits in order to predict vulnerability to cavitation across conifer species. According to our knowledge about conduit widening [46,47,49], anatomical proxies (tangential lumen diameters, CF/mm) may be as well suitable to predict vulnerability to cavitation along tree trunks and branches. We did not investigate different provenances of a given species, so we do not know if a high variability in $P_{50}$ exists among them. Basic wood density and anatomical proxies are reliable on the inter-specific level, but we found no relationships at the intra-specific level for a given provenance of a species (Figure 4). A further step would thus be to test different provenances of a given species, with e.g., different growth characteristics, for relationships between hydraulics and wood anatomy. For such investigations, $P_{25W}$ could be used instead of $P_{50}$ because their intra-specific relationships followed the same trend as the inter-specific ones (Figure 3).

5. Conclusions

The novel proxy $P_{25W}$, i.e., the pressure application that is necessary to result in 25% relative water loss, is regarded as a reliable phenotyping tool for screening conifer species for vulnerability to cavitation, as the relationship between RWL and conductivity loss is assumed to be robust across conifer species. Recently, [34] reported that it was possible to construct 10–25 vulnerability curves/week/person with the air injection method. To determine $P_{25W}$, it is not necessary to measure the hydraulic conductance; therefore, 40–100 relative water loss curves/week/person can be produced, whereby the equivalent of one working day (8 h) is dedicated to sample preparation and determination of the dry weights. We also see a high potential for the proxy CF/mm, i.e., the number of radial tracheid files/mm circumference, (a) as a screening tool for drought sensitivity of wood where samples can be taken “non-destructively” by wood coring and (b) for application in dendroecological studies that retrospectively investigate tree mortality and forest dieback. Determination of the anatomical proxy CF/mm demands only moderate skills in histology and image analysis.
Author Contributions: Conceptualization, S.R.; methodology, S.R., S.N. and K.V.; validation, S.R., S.N. and K.V.; formal analysis, S.R., S.N. and K.V.; investigation, S.R., S.N. and K.V.; data curation, S.R.; writing—original draft preparation, S.R.; writing—review and editing, S.R. and K.V.; visualization, S.R.; supervision, S.R.; project administration, S.R.; funding acquisition, S.R. All authors have read and agreed to the published version of the manuscript.

Funding: “This research was partly funded by the Norwegian Research Council (project “Dieback in Norway spruce”, No. 199403), by the Norwegian Forest Owners’ Research Fund “Skogtültaksfondet”, six regional funds in Norway (Fylkesmannen).

Data Availability Statement: The dataset is available from the corresponding author on reasonable request.

Acknowledgments: Susi Scheffknecht is acknowledged for valuable assistance in the histology laboratory at BOKU University. We thank Helga Amreiter (Pötzleinsdorfer Schlosspark, Vienna, MA 42) for providing plant material of Taxodium distichum. We also thank Hugh Morris for critical reading of the manuscript and for linguistic corrections.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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