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SANTA CRUZ

**PHYSIOLOGICAL LIMITS OF REDWOOD SAPLING RECOVERY
FOLLOWING DROUGHT**

A thesis submitted in partial satisfaction
of the requirements for the degree of

MASTER OF ARTS

in

ECOLOGY AND EVOLUTIONARY BIOLOGY

by

Scottie Herndon Meek

June 2024

The Thesis of Scottie Herndon Meek
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Abstract

Physiological limits of redwood sapling recovery following drought

by

Scottie Herndon Meek

Forests are experiencing dieback due to drought, making it imperative to understand the mechanisms of drought-induced mortality to better predict and manage forests under a changing climate. My research goal was to capture the link between xylem function and cambial viability in *Sequoia sempervirens* saplings under drought. Greenhouse-grown saplings were exposed to water potentials known to cause 50% and 88% loss of water transport due to air expansion in the xylem cells (embolism). These water potentials were identified as levels of mild and severe stress. Xylem water transport and cambial viability were assessed at these targets, and compared against well-watered controls. The cambium was viable under mild drought and able to recover under rewatering regime, but not under severe drought stress. The cambium produces new growth rings each year, so understanding its ability to recover from drought will provide a critical mechanistic link between xylem function and potential for recovery.

This is dedicated to my family;

My research is for the trees...

ACKNOWLEDGEMENTS

My project was supported with funds from the UC Santa Cruz Plant Sciences Award. Many thanks to the greenhouse directors - Sylvie Childress and Laura Palmer - and their amazing staff for growing and managing these saplings. And finally, thank you to my mentors Jarmila Pittermann, Ryan Salladay and the members of Pittermann Lab at UCSC - your guidance and teachings have meant a lot to me :)

INTRODUCTION

Climate related events are impacting our forests around the world. Recent estimates in California suggest over 170 million trees have succumbed to combination of drought and heat events within the past decade (US Forest Services, Axelton, 2019, Cheng et al. 2024). Alarmingly, these include species considered to be drought resilient, highlighting the need to understand current physiological thresholds of tree mortality (Allen et al., 2010, Adams et al., 2017, Hammond et al., 2019). Recent work has identified three key pathways contributing to tree death during a prolonged drought: hydraulic failure, carbon starvation and herbivory attacks (McDowell et al., 2008, Sevanto et al., 2014, Wang et al., 2021). Hydraulic failure, or the inability of the plant to transport water from roots to shoots, is primarily responsible for the death of trees during drought, but water deficit has cascading effects on a tree's carbon budget and by extension, its ability to allocate carbon to defenses, due to stomatal closure, which reduces photosynthesis and carbon availability. The precise threshold beyond which trees die, and below which trees recover from drought is still unknown. We hypothesize that mortality and post-drought recovery is explicitly linked to the health of the vascular cambium. The cambium lies adjacent to the xylem, and is directly dependent on its hydration to produce new xylem and phloem tissue. No study has linked hydraulic failure, tree death and recovery potential to the cambium. My work presents the first attempt to

connect low water potentials, hydraulic failure and cambial viability to drought-induced mortality.

Prior research has established hydraulic failure as the main driver of drought-induced mortality in trees (McDowell et al., 2008, Sevanto et al., 2014, Anderegg et al., 2015). Failure to transport water occurs when xylem water potentials drop below some species-specific threshold and a phenomenon known as embolism occurs, which describes the process of air seeding into xylem conduits that expand and ultimately block water flow (Tyree & Sperry, 1989). Trees with a large fraction of embolized xylem suffer canopy dieback, and in many instances, mortality (Allen et al., 2015). Woody plants do not refill their embolized conduits, so efficient water transport resumes after the growth of a new ring of wood (Brodribb & Cochard, 2009, Choat et al., 2016, Hammond et al., 2019). The production of the new growth ring depends on the viability of the cambial layer, so recovery from water deficit is possible if the vascular cambium remains hydrated and thereby viable.

The vascular cambium is a layer of meristematic cells that divide peri- and anti-clinally to produce secondary xylem and phloem (Spicer & Groover, 2010). Research within the past two decades that examined cambial mortality has been mainly limited to anatomical analyses that rely on standard fixation, embedding and sectioning methods (Gričar et al., 2009, Gričar et al., 2014, L. Wang et al., 2020, Li et al., 2022). Traditional microscopy is time consuming, expensive, and relies on experts for interpretation. However, a recently developed staining assay has improved our potential to examine the viability of the vascular cambium (Salladay and

Pittermann, 2023). The assay uses the neutral red tissue stain, which is transported by the tonoplast proteins in living cells such that functioning vacuoles will appear pink and dead cells will show little to no color. Stained vacuoles can be discerned in fresh sections allowing for efficient image analysis and thus data collection under experimental conditions and *in situ*. When applied consistently, this assay can discern cambium viability in response to a range of stresses, including fire, drought stress and other abiotic/biotic impacts. My project uses this assay to capture the association between cambial viability, drought stress and recovery.

My project focused on *Sequoia sempervirens* (Lamb ex. D. Don) saplings. This species is endemic to California and Oregon coasts and our current forests suffer widespread, drought-induced mortality events throughout its native range (van Mantgem et al., 2009, Breshears et al., 2005, Fettig et al., 2019, Axelson et al., 2019). Redwood is an economically important conifer so I was able to use saplings from a local nursery to conduct a greenhouse drought experiment. Understanding the mechanisms of tree mortality and possible recovery is essential for effective forest management. Hence, I sought to answer these main questions: 1) Is cambium viability linked to hydraulic function in redwood saplings under increasing drought conditions? 2) How does the cambium respond to rewatering at two key water potentials? 3) What can this tell us about the relationship between xylem function and vascular cambium? I hypothesized that hydraulic transport and cambial health would not be significantly affected in saplings under mild treatments, however severe drought will surpass the dehydration threshold, thereby preventing recovery from

rewatering. My results provide empirical evidence for the connection between dehydration thresholds and tree recovery from drought-induced mortality.

METHODS

Experimental Overview

The goal of my experiment was to determine how vulnerable xylem function and cambial health are to increasing drought conditions. Hydraulic failure was determined with two initial experiments that used two different methods of quantifying sapling vulnerability to embolism - the centrifuge method and bench drydown. Xylem conductivity readings were plotted against water potential to produce a vulnerability curve, which I then used to estimate target water potential at which 50 and 88% of conductivity was lost due to drought-induced embolism (hereafter called P_{50} and P_{88}). At -2.5 and -5.5 MPa, respectively, these values have been identified as critical thresholds of tree stress and tree mortality (McDowell et al., 2008, Brodribb & Cochard, 2009, Choat et al., 2016, Hammond et al., 2019).

In the summer of 2023, I conducted a greenhouse drought experiment with 32 saplings to capture hydraulic failure and cambium viability at mild (P_{50}) and severe (P_{88}) water stress. Figure 1 shows a visual timeline of the sampling design, including initial harvests and rewatering regime. For drought treatment and control plants ($n=8$), the axial region of the main stem was removed 30 cm from the base of the sapling. Three subsections were harvested for hydraulic and cambium measurements: the axial 5 cm were perfused with stain to identify functional xylem area, the next 10

cm were harvested for measurements of xylem conductivity, and the remaining 1-2 cm were harvested for cambium viability staining. Once cut, the basal regions of the same saplings were placed under a week-long rewatering regime to assess recovery potential. The same measurements were repeated on the basal half of the main stem. Xylem dye perfusion cross sections and cambium microscopy sections were photographed and analyzed using standard software, followed by statistical analyses using R software (v4.3.3; R Core Team 2024).

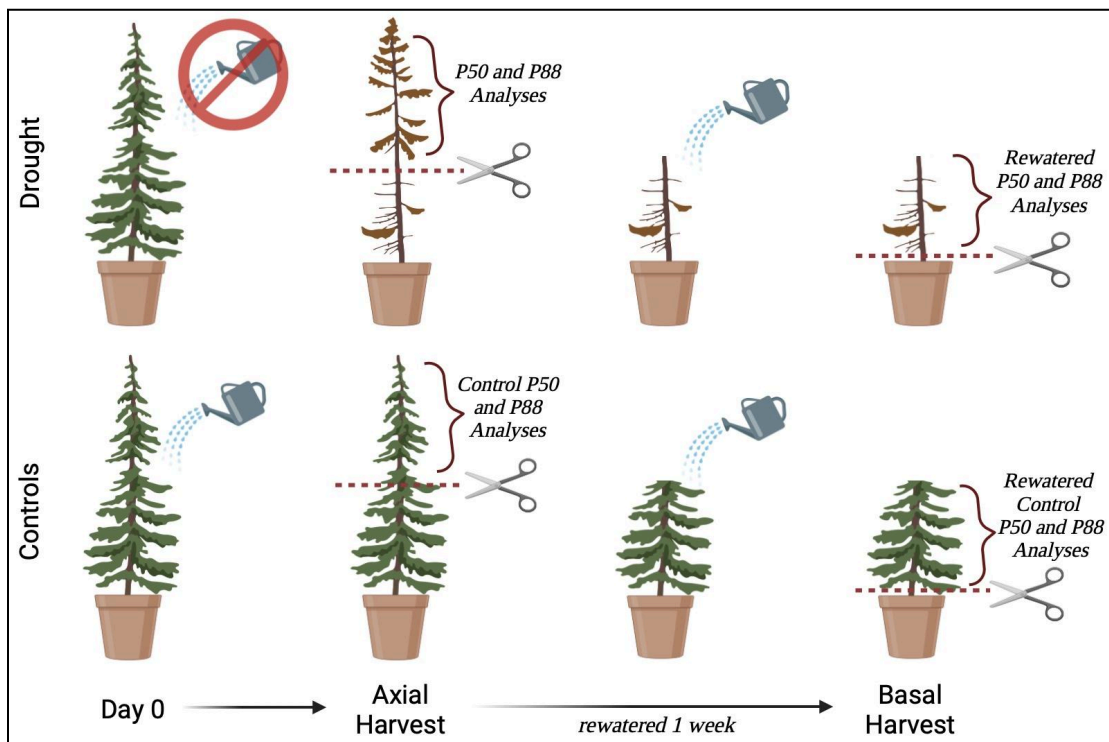


Figure 1: Experimental Timeline. Schematic illustrating harvesting regime for saplings allocated to drought and control treatments. Time from day zero to axial harvest varied per sapling depending on how many days it took to reach target P₅₀ and P₈₈ values.

Seedling Preparation

Sequoia sempervirens seedlings were acquired from a nursery (The Jonsteen Company, McKinleyville, CA) in May 2022. Seedlings were transplanted into one-gallon pots in the UC Santa Cruz greenhouse using high porosity soil (ProMix HP, Premier Tech Horticulture). Distribution on benches was randomized biweekly to avoid localized microclimate variation. To ensure growth for all seedlings, soil saturation was maintained daily or every other day. Stakes were inserted as needed to support the seedlings as they matured into saplings. Greenhouse conditions remained constant at average room temperatures of 66 °F and relative humidity of 72%, throughout the entire growth period.

Determining Critical Levels of Drought Stress: Centrifuge and Bench Dry-Down Curves

To determine the trend in percent loss of conductivity to increasing drought stress, we established a vulnerability curve using the centrifuge method in Fall of 2022 (Alder et al, 1997). Predetermined spin rates of the centrifuge equate to specific levels of xylem tensions that occur in nature under drought. Eight saplings were selected at random and 14.2 cm sections were prepared from the central stem. Stems were spun for four minutes at increasing speeds to mimic the water potential associated with acute drought stress. After each spin, stem flow rates were measured using a standard pressure head approach whereby final hydraulic conductivities (K) were then captured by a flow meter ($\text{mg mm s}^{-1} \text{ kPa}^{-1}$; SLI-1000, Stäfa, Switzerland)

and converted to conductivity values with R code (v4.3.3; R Core Team 2024). This was repeated for all predetermined xylem tensions (-1 to -10 MPa) for all 8 stems, or until stems reached zero conductivity. The final vulnerability curve was analyzed using the `fitplc` R package (Duursma and Choat, 2017) with a Weibull function (Figure 2).

A follow up pilot experiment with saplings at the estimated P_{50} value (xylem water potential = -4.5 MPa) showed that the centrifuge method overestimated sapling resistance to cavitation. Hence, a manual dry down experiment was conducted in February 2023 to quantify *in vivo* water transport in the potted saplings under conditions that mimicked drought stress. Water was withheld from 36 saplings for 33 days, and three saplings were harvested every three days to examine water potential (PMS INstruments, Albany OR). Samples were randomly selected and bagged for one hour to stop transpiration and thus equilibrate the water potential throughout the plant. Average water potentials were measured from 3-5 branch segments per plant using a pressure chamber instrument (Model 1505D, PMS Instrument Company, Albany, OR, USA). Conductivities were measured for each sapling and plotted against corresponding water potential value to establish a conductivity curve. Final curve was analyzed using the `fitcond` R package (Duursma and Choat, 2017) with both Weibull and Sigmoidal model fits (Figure 2). This dry-down curve provided the P_{50} and P_{88} water potentials for the core drought stress experiment, described below.

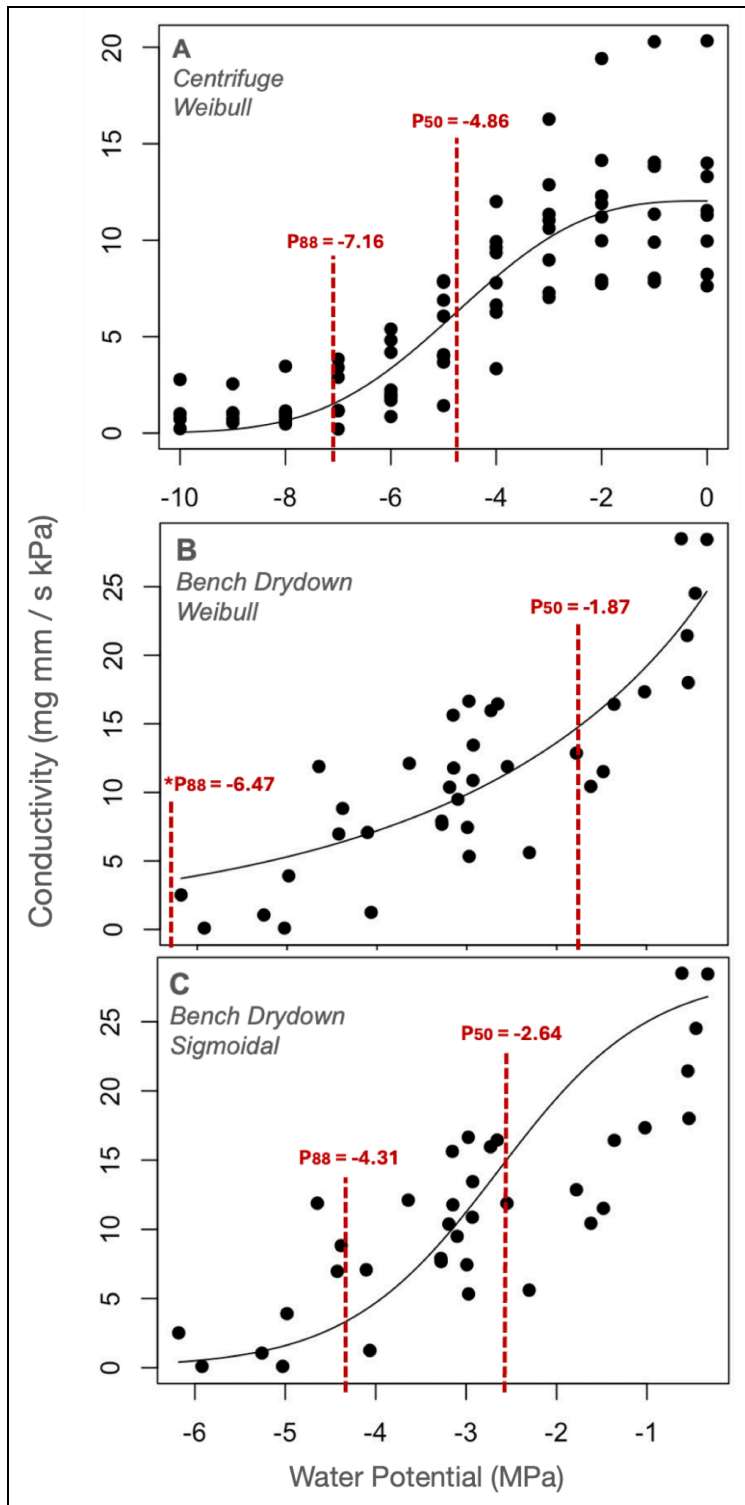


Figure 2:
Vulnerability Curves.
Three analytical approaches were used to examine sapling hydraulic failure in response to drought. (A) The centrifuge-generated vulnerability curves fit with a Weibull function, (B) bench drydown method fit with a Weibull function, and (C) bench drydown fit with sigmoidal function. Red lines show the estimated water potential at which saplings lose approximately 50% and 88% for each respective graph.

The Greenhouse Drought Experiment

The greenhouse dry-down experiment lasted approximately four weeks. 32 saplings were randomly assigned to one of 4 treatment types: P₅₀, Control P₅₀, P₈₈ and Control P₈₈ (n = 8). Target drought stress for the P₅₀ and P₈₈ treatments were determined from the bench drydown method above, and estimated as -2.5 MPa and -5.5 MPa, respectively. These target values are the average values between the Weibull and Sigmoidal fit (Figure 2, B and C) to account for variation between lines of best fit. Control plants continued to receive daily watering, whereas drought treatment plants were moved to a different table and stopped receiving water until target drought stress was reached. Water potential measurements were taken using a pressure chamber on saplings that were bagged for 1 hour to stop transpiration and establish water potential equilibrium within the sapling. Immediately when target drought stress was achieved, axial harvests were made to assess hydraulic function and cambial viability (Figure 1). The distal 5 cm of all saplings were harvested from the central stem and trimmed down with razor blade while fully submerged underwater to prevent introduced embolism. The distal region of the stem was wrapped in parafilm and attached to a dye perfusion apparatus. Following established methods (Pratt & Jacobsen, 2018), a 0.1% aqueous solution of crystal violet was pulled through stem segments at approximately 6-8 kPa. Crystal violet stains non-embolized conduits that are transporting water, thus the amount of blue stain is an estimate of the area of functional xylem. Samples were monitored every 15 min until crystal violet was visibly collecting in the tubing section distal to the stem. For severe drought treatment

samples, monitoring was stopped after two hours if no stain was visible after that duration (suggesting xylem tissue was mostly embolized and unable to transport water). Cross sections were made into the center of the segment (approx 2.5 cm from the ends) and photographs were captured using an iPhone camera (model 11, 2019, Apple Inc) stationed 6 cm away from the sample at 1.8x magnification. Xylem areas were analyzed using Image J and the fraction of functional xylem was calculated as the total stained area over the total xylem area, excluding the area of the pith for both.

After functional xylem measurements, the next 12-14 cm segments were harvested from the central stem for measurements of conductivity (K_{native}). Stem segments were then trimmed down to 10 cm under water to prevent introduced embolism. Side branches were removed and parafilm was wrapped over any openings along the main branch to prevent dehydration. Stem diameter measurements were taken by averaging the diameter of basal and distal ends of each segment. Final conductivity values were measured using the same methods described earlier using the hydraulic apparatus and flow sensor capture converted to values in R software (v4.3.3; R Core Team 2024).

Cambial Viability

Cambial viability was measured using Neutral Red stain assay (Salladay & Pittermann, 2023). I used a microtome (Lab-Microtome, Swiss Federal Research Institute, Switzerland), to prepare tangential longitudinal sections, 80 μm thick of each stem. These sections were stained using the Neutral Red solution for 20 minutes

(0.01% solution of Neutral Red in a 20 mM phosphate buffer pH 7.5, 600 mM KNO₃, and 1 mM CaCl (Basham & Bateman, 1975)), and then rinsed (solution of 20 mM phosphate buffer pH 7.5, 600 mM KNO₃, and 1 mM of CaCl). Sections were prepared on standard microscopy slides and images were captured using a compound light microscope (Leica, Wetzlar, Germany) for a total of ten images of each stem from top and bottom orientations. ImageJ software was used to analyze the images and quantify living cell viability. Neutral red stain accumulates in the vacuoles of living cells, thus we can estimate the amount of viable cambium by the proportion of vacuoles stained within a selected area (300 - 350 thousand μm^2 ; Figure 3). Final cambial viability was calculated as the average of five, randomly selected images per stem.

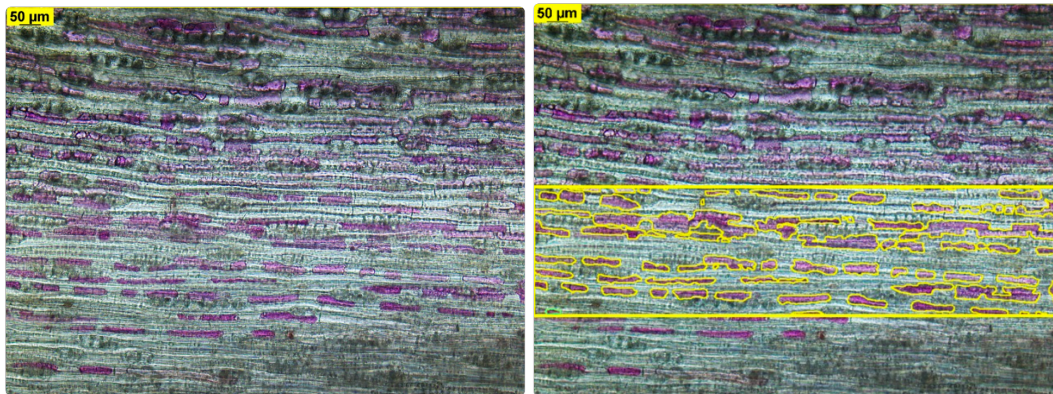


Figure 3: Vascular Cambium Microscopy. Microscopy image of vascular cambium on Image J analysis software, showing with (left) and without (right) standardized overlay at 10x magnification.

Statistical analysis

All statistics were calculated using R software (v.4.3.3, R Core Team, 2024).

Data was tested for normality and homogeneity of variance using the Shapiro Wilk

and Bartlett test included in the standard R package. Data were not normally distributed, thus a Kruskal-Wallis ranked sums test was performed to determine if hydraulic function and cambium viability varied across treatments. A standard Wilcoxon post-hoc test was used for analysis between drought and control groups, whereas a pairwise Wilcoxon post-hoc test was used for analysis between the basal rewatering groups across all control and drought plants. P-values were interpreted with a Bonferroni adjustment.

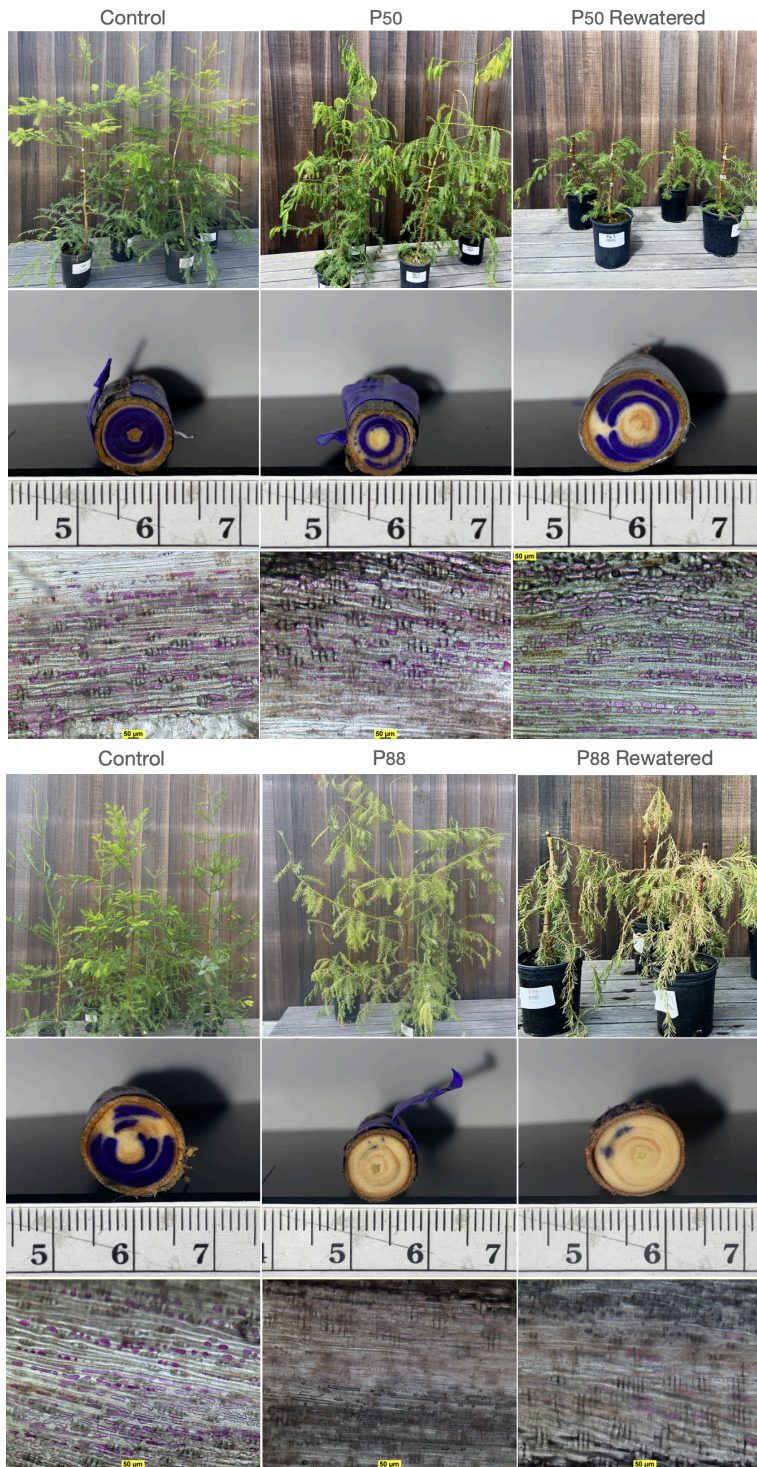


Figure 4: Observations. Example photos of drought experiment treatments and standard imaging. Columns denote the treatment group, and rows show sample images of the plant samples, cross section of functional xylem stainings and vascular cambium microscopy images, respectively in vertical order. Images from the rewatered controls treatment are not shown, but were harvested with the same regime as described in the methods section.

RESULTS

Estimated target water potential values from conductivity curves varied across the two methods (Figure 2). The centrifuge method produced a P_{50} of approximately -4.8 MPa, whereas the bench drydown method produced a P_{50} closer to -2.5 MPa (an average of the A) Weibull and B) Sigmoidal fits, Figure 2). A P_{50} value of -4.8 MPa does not accurately represent the biological response of the sampling, so I opted to use P_{50} and P_{88} values from the bench drydown method average of the Weibull and the Sigmoidal fits as the target water potentials for the *mild* and *severe* drought treatments, respectively.

P_{50} values were reached in saplings left 5-7 days without water, while the P_{88} values were reached between 22-27 days. Drought treatment saplings were harvested within 10% of the target P_{50} and P_{88} values (Figure 5). Water potentials for the P_{50} and P_{88} drought treatment plants were significantly different from their respective control groups ($P < 0.0005$). Rewatering returned water potentials of P_{50} saplings back within the range of fully hydrated control saplings (rewatered P_{50} = -0.45 ± 0.194 MPa, $P = 0.505$). However the saplings did not recover water potential values with rewatering after severe drought stress (rewatered P_{88} = -4.5 ± 1.83 MPa, $P < 0.0005$). Three of the rewatered P_{88} saplings returned to water potentials between -1.5 and -2.5 MPa, but this may be due to passive conductance via xylem wicking rather than functional xylem transport. Furthermore, pairwise Wilcoxon test did not detect a significant difference between P_{88} and rewatered P_{88} ($P = 0.72$).

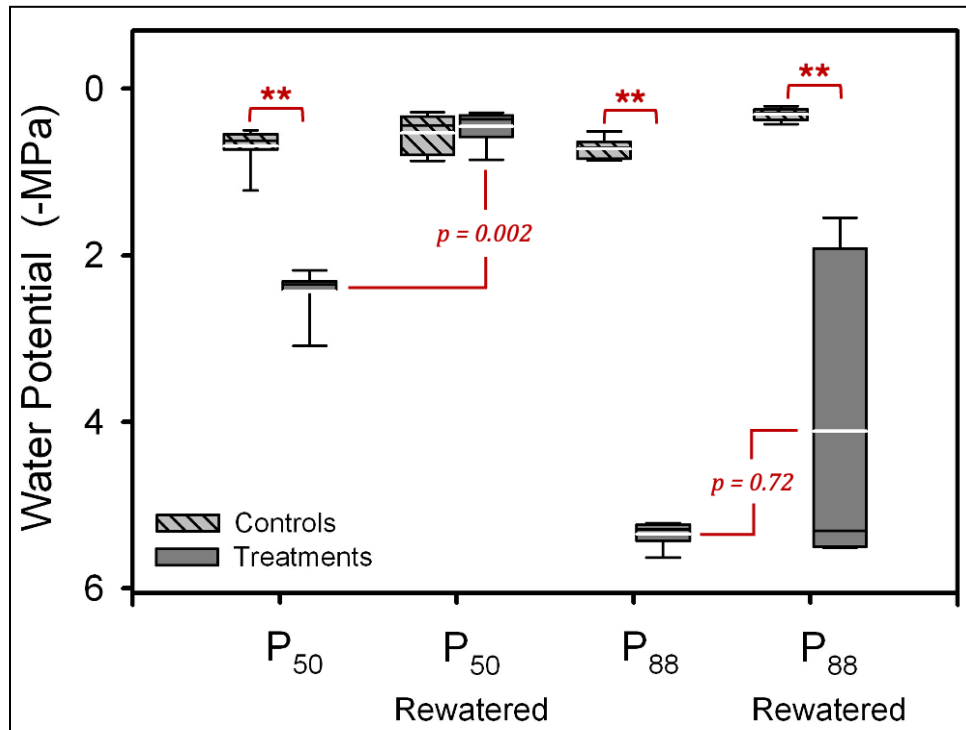
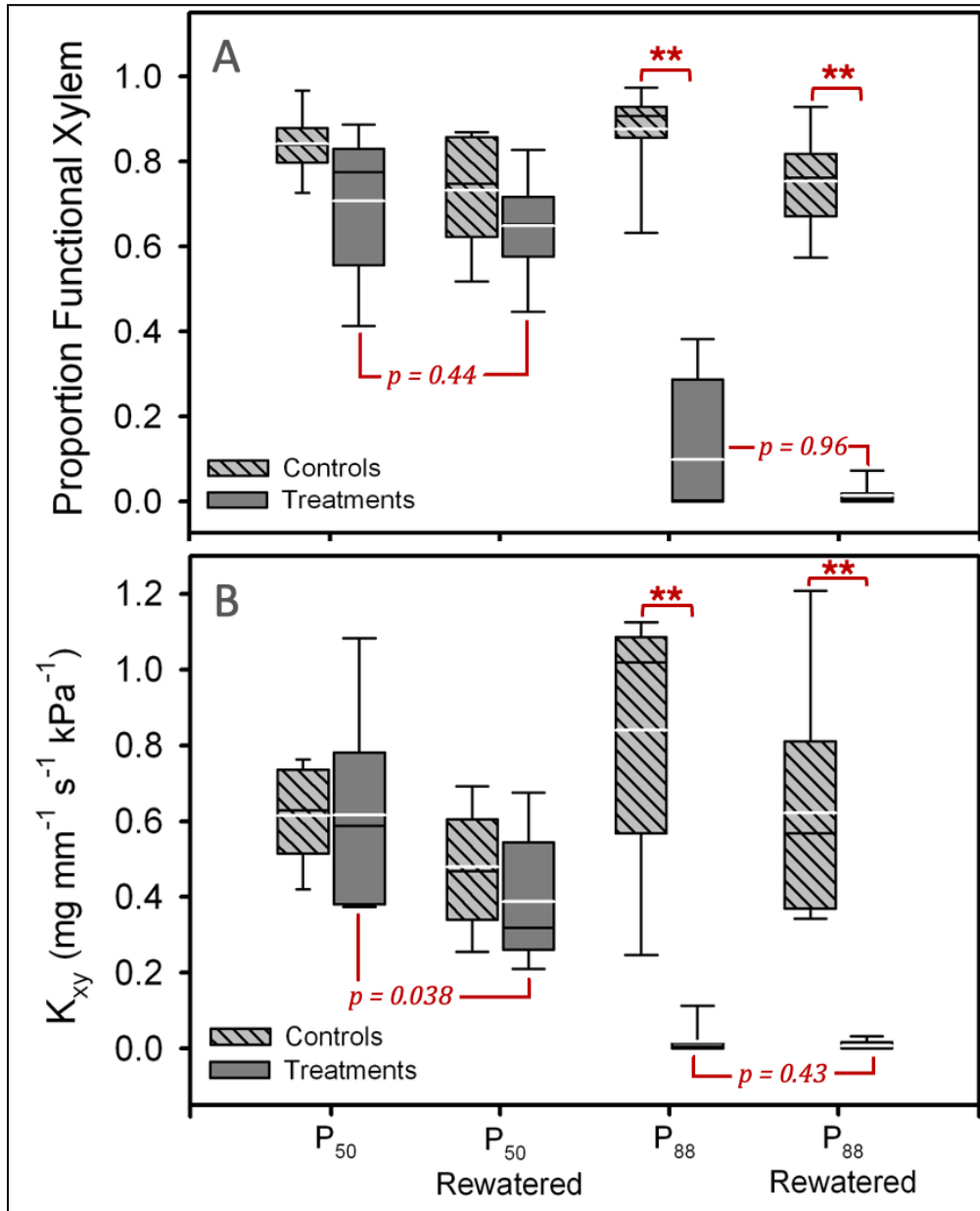


Figure 5: Water Potentials. Water potential measurements across treatments taken with a pressure chamber. Asterisks denote significant differences between relative drought and control treatment groups ($P < 0.0005$). Red p-values are from pairwise wilcoxon test for drought and rewatered drought saplings.

Hydraulics

Xylem function and rates of water transport were consistent with the loss in water potential (Figure 3). The fraction of functional xylem area stayed relatively constant across all fully hydrated controls and mild drought treatment plants (Figure 3A, $P > 0.1$). Severe drought treatment reduced the functional xylem fraction to only 10% of total xylem area, relative to the control plants in which an average of 88% of the xylem was functional ($P = 0.0009$). Rewatering had no effect on the fraction of functional xylem across all treatments ($P > 0.1$). Generally speaking, rewatering

failed to recover the functional xylem fraction across all drought-stressed saplings, although this observation is not statistically significant.



Trends in conductivities resonate with the functional xylem data (Figure 3B). The conductivity in plants subjected to the P₅₀ drought treatment was similar to the well-watered control plants. When both the control and P₅₀ drought stressed plants were rewatered, conductivity was again similar. However, rewatering did not fully rehydrate the xylem of plants subjected to mild drought ($P = 0.038$; Figure 3B). Plants exposed to the severe P₈₈ drought lost much of their capacity to transport water ($P < 0.03$) and did not recover after rewatering ($P = 0.43$). Taken together, the failure to recover embolized xylem after rewatering suggests that once embolism is introduced into the xylem, the conduits do not refill and water transport is permanently blocked.

Vascular Cambium Viability

Results from the neutral red staining assay showed the most variability (Figure 4). Mild drought treatment plants decreased their fraction of viable cambium significantly from control values ($P = 0.0002$). However, cambium fractions of these droughted plants returned to similar values of fully hydrated controls upon rewatering (rewateredP₅₀ = 0.342 ± 0.044 , $P = 0.16$). Saplings from the severe drought treatments decreased the fraction of viable cambium significantly from control plants, such that approximately 5% of the cambium was viable within axial harvest and stayed below 10% viability upon rewatering (P₈₈ = 0.057 ± 0.83 ; rewateredP₈₈ = 0.071 ± 0.069 , $P = 1$).

It is important to note the anomaly that occurred with the rewatered control plants for the P₈₈ group (Figure 7, rewateredP₈₈, light gray lined bar). The fraction of viable cambium was significantly lower when compared to the other 3 control groups (Control rewateredP₈₈ = 0.2 ± 0.026, $P < 0.01$), more similar to the average value of the P₅₀ mild drought treatment group (P₅₀ = 0.24 ± 0.062). We currently have no explanation for this discrepancy, aside from the potential impacts from axial harvesting method or volatile release from neighboring stressed plants during rewatering period.

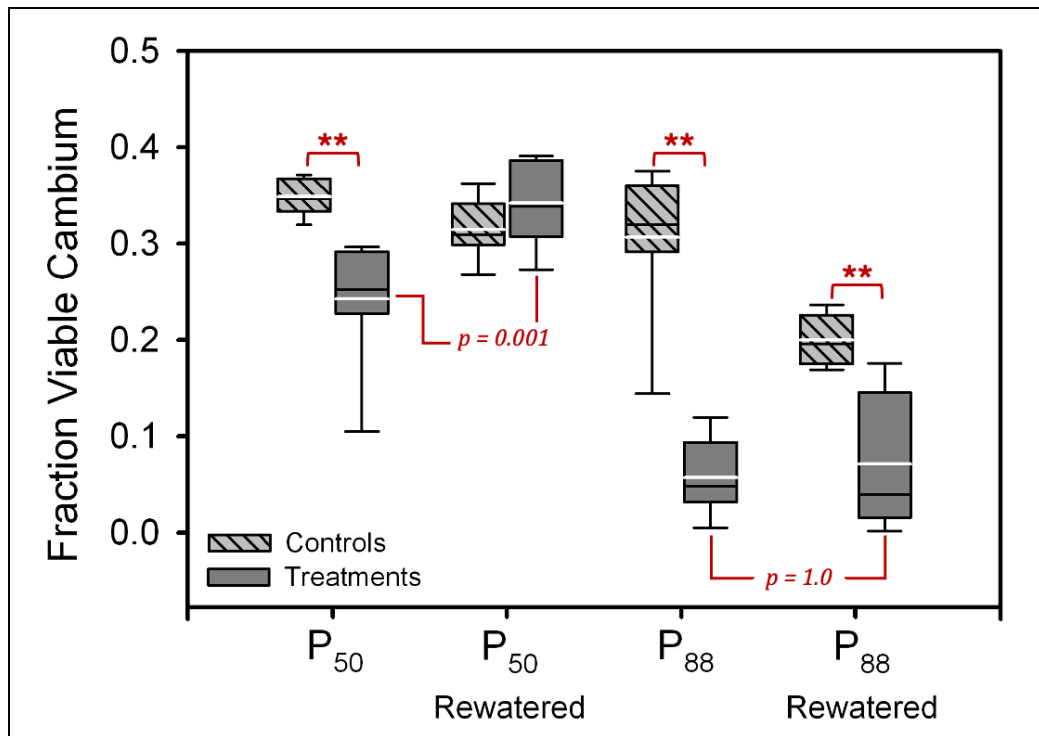


Figure 7: Cambium Viability. Cambium viability across treatments calculated from Neutral Red staining assay. Asterisks denote significant differences between relative drought and control treatment groups ($P < 0.0005$). Red p-values are from pairwise Wilcoxon test for drought and rewatered drought saplings.

DISCUSSION

My work provides evidence for an empirical association between hydraulic failure, cambium viability and recovery potential of *Sequoia sempervirens* saplings following drought stress. Severe drought not only embolizes the xylem, but it also kills the vascular cambium and precludes recovery after rewatering. By contrast, saplings can recover from mild levels of drought stress because the vascular cambium remains viable. These results provide further insight into the mechanism of drought-induced tree mortality.

The centrifuge method did not accurately capture loss in conductivity in *S. sempervirens* saplings. The estimated P_{50} value from the centrifuge-generated vulnerability curve ($P_{50} = -4.5$ MPa) was incorrect, and implied that the saplings were more drought resistant than they were in reality (Figure 1). The manual, bench drydown method produced a more accurate P_{50} value ($P_{50} = -2.5$ MPa). This value appears to be more indicative of the *in vivo* vulnerability of *S. sempervirens* saplings. The centrifuge method can accurately capture species vulnerability to drought-induced embolism but it is essential to understand its limitations, and to consider the possibility that even within predictable systems, such as conifers, the vulnerability curves will vary depending on the plant material (Pittermann et al., 2021, Salladay and Pittermann, 2023). Other methods exist, but they too come with penalties including the need for larger sample size and ample time for analyses (Brodribb & Cochard, 2009, Adams et al., 2017, Brodribb et al., 2017).

My results provide empirical evidence of irreversible hydraulic failure with severe drought. Increasing drought stress resulted in decreased hydraulic function. Functional xylem and water transport were slightly impacted with our mild drought treatment and were able to maintain similar functional values compared to control saplings that were fully hydrated (Figure 3). However, the saplings subjected to severe drought had xylem that was almost completely embolized and did not refill after rewatering. These results suggest that the target P_{88} value surpassed the threshold value of xylem transport capacity. Furthermore, the decrease in both conductivity and proportion of functional xylem across all initial treatments and their rewatered, basal regions suggests irreversible blockage to xylem transport may have occurred with the distal harvest. This harvesting cut to the central stem introduced a small degree of embolism into xylem conduits that cannot recover transport until secondary xylem is added. Future studies would benefit from sampling branch segments instead of central stem to avoid this irreversible blockage.

The cambium is viable at P_{50} water potentials but not at P_{88} . My data support the hypothesis that cambial viability is linked with hydraulics, specifically, hydrated xylem in proximity to the cambium. Some threshold water potential is required to maintain a certain degree of hydration in order for the cambium to remain viable, thus facilitating its role in the production of new, secondary xylem. This, by extension, provides the link between hydraulic failure, cambium function and tree recovery in the context of drought. Further research into the viability of cambium at intermediate

values of drought stress alongside hydraulic failure is needed to identify the “point of no return” (McDowell et al. 2008; McDowell et al., 2022).

Measures of vascular cambium viability were consistent, but variable. This assay is nuanced and requires some microscopy experience and strong knowledge of tree anatomy. However, it is the first method to rapidly and efficiently quantify cambial health. We are currently investigating how to further standardize image analyses and increase the accuracy of staining procedures in order to allow future studies to utilize this assay. Currently, other methods for analyzing the vascular cambium have been limited to anatomical counts or qualitative checks - such as counting the number of cambium cells or bark removal to directly examine the cambium (Smith et al., 2011, Gričar et al., 2014). Further comparisons of the neutral red assay alongside cambium bark removal could allow us to increase the accuracy across these different methods. However, this assay is achievable with minimum resources and time, and can be applied across multiple sample types, including cores or roots.

My results provide insight into a plant tissue that has an important role in the recovery potential of trees under drought. Further research into the thresholds of this tissue alongside xylem and other tree mortality features, such as canopy coverage or leaf traits, would help strengthen our understanding of the physiological mechanisms of tree mortality. Along with other research, my work can help provide tools to aid in management and conservation of drought-stricken forests around the world.

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