

**Transform or Perish:
Changes in Leaf Venation in Response to Punctures**

David S. Zhong

ABSTRACT

Leaf venation systems are integral in distributing nutrients and water to photosynthetic cells in angiosperms. Uniquely in angiosperms, major and minor veins can be distinguished, where major veins receive nutrients from the petiole and distribute it to minor veins. Warmer climates are more favorable to chewing insects, and the resulting herbivory has led to a decrease in plant growth. Biotic factors such as herbivory from chewing insects potentially inhibit or increase the vulnerability of the successful transportation of water and nutrients through leaf veins, which are more susceptible to environmental drivers and changes. This study evaluates how leaf venation systems in angiosperms respond to puncture wounds by quantifying leaf vein density and how petiole growth affects leaf vein density. I experimented on two sets of *Cercis canadensis* (redbud) tree leaves and examined them using a digital microscope. After analyzing through image processing, I found the sets of leaves receiving treatment noticed a significantly higher leaf vein density and more succinctly formed vein loops. Additionally, leaf vein densities closer to the puncture site responded with larger leaf vein densities. Finally, the differing petiole growth and leaf vein density followed a U curve with the maximum density at a difference of 0.12 mm. These findings point to their adaptability and the importance of leaf venation systems when faced with adversities such as damage from insect herbivory.

KEY WORDS

leaf vein density, petiole, puncture, herbivory, plant growth

INTRODUCTION

In recent decades, climate change, which has led to a larger increase in insect pests due to proliferation more favorable in warmer climates, has significantly reduced crop yield (Moro and Soares 2020). In the last century, an estimated 0.6° Celsius increase has resulted in an estimated 52% decrease in forest density (Boisvenue and Running 2006). Climate change ultimately has devastated smallholders/traditional family farming communities and wreaked havoc among ornamental plants (Altieri and Koochafkan 2008).

The increase in the prevalence of locust swarms can be attributed to the effects of climate change, with warmer temperatures propagating more favorable circumstances for pest species (Peng et al. 2020). Fundamentally, insect grazing rates affect both young and mature leaves equally, with the percentage change in the amount of holes against the percentage change in total leaf area to not be significantly different (Coley 1980). Nevertheless, the holes induced in young leaves caused by insects can present a detrimental challenge to the growth and survival of plants. As the leaf matures, the size of the hole increases, as well as altering the shape of the leaf, which can inhibit overall energy production capability in a plant's system (Lowman 1987). Increases in variability in climate can negatively affect locations worldwide by increasing populations of herbivorous insects, which may permanently stunt horticulture.

Leaf venation systems are critical to a leaf's survival by providing nutrients to the cells that undergo photosynthesis (Boer et al. 2016). The hydraulic conductivity capacity—the efficiency for water to disperse throughout the leaf—is linked to the maximum photosynthetic rate, indicating the ability for water to flow throughout venation systems to be a significant predictor of plant growth (Brodribb et al. 2007). Although there are a variety of leaves with unique shapes, patterns, and sizes, "geometric rules" govern how veins are arranged throughout a leaf. This symmetry is essential as it conveys the optimal formation of leaf veins throughout various plants in contrasting environments (Zhang et al. 2018). Peculiarly in angiosperms, major and minor veins can be distinguished, where major veins receive nutrients from the petiole and distribute it to minor veins (Feild and Brodribb 2013). The complex vein systems throughout plants play a vital role in sustaining the individual leaf and collectively supporting a plant as a whole by providing a structure for transporting nutrients.

Herbivory can interfere with efficiently transporting nutrients and water through leaf veins (Zagrobelny 2013). Fortunately, leaf venation systems are unique as they can quickly adapt to damages and fluctuations, either bypassing the injury, creating strength and thickness in the used veins, or creating more minor vein pathways (Katifori et al. 2010). When damaged, the auxin flux bifurcates, allowing vein tips to attach to more veins, constructing more vein loops (Feugier and Iwasa 2006). Additionally, leaves become thicker because blocked pathways in leaves lead to a higher hydraulic conductivity through undamaged alternative pathways (Scoffoni et al. 2011). Because plants are remarkable in their ability to adapt to environmental stressors, learning about the mechanisms that allow plants to survive may help develop solutions for increasing agricultural yields even with environmental uncertainty. Little research has compared the leaf vein densities at different leaf locations, and fluid mechanics have only been theorized. Furthermore, plant parts such as the petiole govern the max hydraulic capabilities of leaves and may predict the overall efficiency of water transport in leaf veins. However, few studies are analyzing the relationship between the petiole diameter and the impact of leaf puncture on leaf vein density.

In this study, I explore the following question: How do leaf venation systems in redbud leaves (*Cercis canadensis*) respond to damage to veins? In particular, I examine (1) How does a puncture in the leaf's midrib affect the density of leaf veins compared to leaves not punctured? (2) Are leaf vein densities different in areas farther away from the puncture site? (3) How does a leaf vein density vary across punctures at different petiole diameters? Leaf vein density and leaf vein loop structure characteristics directly reflect how the veins change in new conditions and thus can elucidate the resiliency of leaves. I expect leaf vein densities and the number of incomplete leaf vein loops to be higher in punctured leaves than in non-punctured ones. Likewise, I hypothesize that leaf vein densities are higher near the puncture site of the leaf rather than farther away from the puncture site. I also expect that leaf vein density to be highest at leaf punctures at a smaller petiole diameter because smaller leaves tend to have greater leaf vein densities.

METHODS

Study organism: *Cercis canadensis*

Cercis canadensis is an ornamental landscape tree more commonly known as the Eastern Redbud. Mature plants reach a height of 15-30 ft. and bloom pink clusters of flowers during the spring, which eventually transition into heart-shaped leaves during the summer (Figure 1, Figure 2). The flowers bloom in groups of four to ten, which later flatten into leguminous, bean-like seed pods that mature in summer, with each pod containing between six to twelve seeds (Missouri Botanical Garden). *Cercis canadensis* is mainly an ornamental perennial tree with deciduous characteristics, shedding its leaves annually during the late fall.



Figure 1. This is a redbud (*C. canadensis*) tree in suburban Salt Lake City, Utah (Salt lake County), in early June.



Figure 2. Image of a trial *C. canadensis* leaf with midrib punched to simulate herbivore damage. Some of the surrounding leaves were damaged by insects (such as a caterpillar or katydid).

The primary consumers of *C. canadensis* consist of insects such as weevils, webworms, treehoppers, leafhoppers, spider mites, caterpillars, and scale (Lady Bird Johnson Wildflower Center). *Cercis canadensis* is a native species to Eastern North America but can be found all throughout North America in California and Mexico as well (Donselman and Flint 1982).

Morphology: *Cercis canadensis*

Cercis canadensis bears leaves that typically reach a width of about 3-5'' across. *Cercis canadensis* has seven major veins that distribute nutrients to its minor veins—secondary and tertiary minor veins (Figure 3).

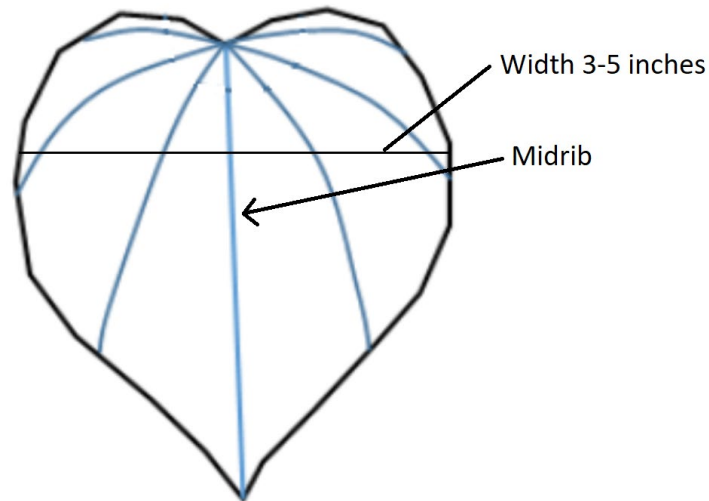


Figure 3. *Cercis canadensis* has seven major veins. The middle vein is called the midrib.

Study site

I conducted this experiment in Salt Lake City, UT, between June 26th and July 11th, 2021, at an altitude of 1,500 meters above sea level. The Salt Lake climate is often dry and desert-like, with little to no moisture in the air and temperatures that fluctuate from 11° Celsius (night) to 34° Celsius (day). Automatic sprinkler systems in the morning ensured adequate and even water distribution.

Data collection

I studied leaf venation systems on two sets of 16 leaves from a singular *C. canadensis* (redbud) tree, where I removed leaves at the end of the study, imaged, and digitally analyzed each trial. To ensure the range of sun exposure would not influence the test, I chose leaves everywhere around the tree. I chose the leaves in pairs, with the punctured leaf paired with a control leaf with similar characteristics in terms of location, size, and petiole diameter. The first set of 16 leaves acted as a control group receiving no treatment, with the second set receiving hole punches in its midrib to simulate insect feeding damage (Figure 2). Although insect damage is typically unevenly distributed throughout a leaf, I punched all leaves at the midrib (central vein) using a hole puncher for consistency. I marked each punctured leaf with red-colored string and the control group with green-colored string at the leaf's petiole. I measured the petiole diameters of punctured leaves with calipers to the hundredth degree of a millimeter.

At the end of two weeks, I collected all the leaves by cutting where the petiole meets the branch. To prevent differences from desiccation, I immediately imaged all cut leaves by taking an (8.5 by 11 inches) black and white printer scan with an Epson 3640 Scanner to calculate surface area. I used these black and white scans to calculate the surface area of the leaf by calibrating it with a five by five-centimeter image of a square (Figure 5). I also imaged all leaves with microscopic images in four quadrants of the leaf (Figure 4) with a digital Celestron USB Microscope, 40X-1000X Digital Microscope 3 in 1 PCB at 25x magnification. After the imaging was completed, I discarded the leaves.

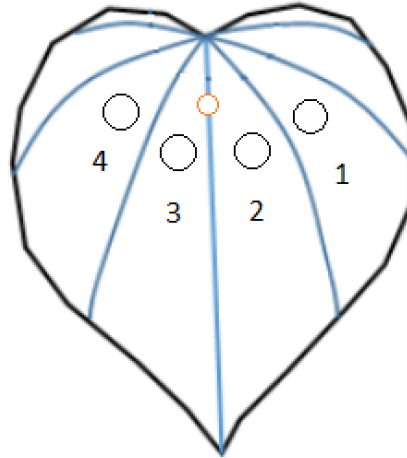


Figure 4. Black circles approximate where microscopic images were taken, with quadrants 1 and 4 being further away from the puncture site and 2 and 3 sharing the same vertex. The orange circle represents where the hole punch position on punctured leaves, which were about 1.5 cm away from the end of the petiole. Microscope scans were taken beyond the puncture site, which will vary on the size of the trial.



Figure 5. Size of the leaf (surface area) pixels counted and ratio of 5 by 5 square to find surface area in cm.

Image editing

I assessed the trials of leaf venation systems using leaf vein density (LVD) and the number of leaf vein loops (LVL). I analyzed the microscopic images using Gimp 2.10.24, which is an image editing software (The GIMP Development Team 2019). I also qualitatively noted the difference in size and shape along with incomplete vein loops just beginning to form. To calculate leaf vein density, I converted the green-yellow microscope image to grayscale and

finally to black and white scale using Gimp 2.10.24 (Figure 5). I then counted the pixels using the histogram statistics to receive a value of the number of black pixels against the number of white pixels (black pixels reflected in the minor veins). Other studies on leaf venation systems used similar techniques when gathering the number of leaf vein loops (Katifori et al., 2010) and leaf vein density (Boyce et al., 2009).

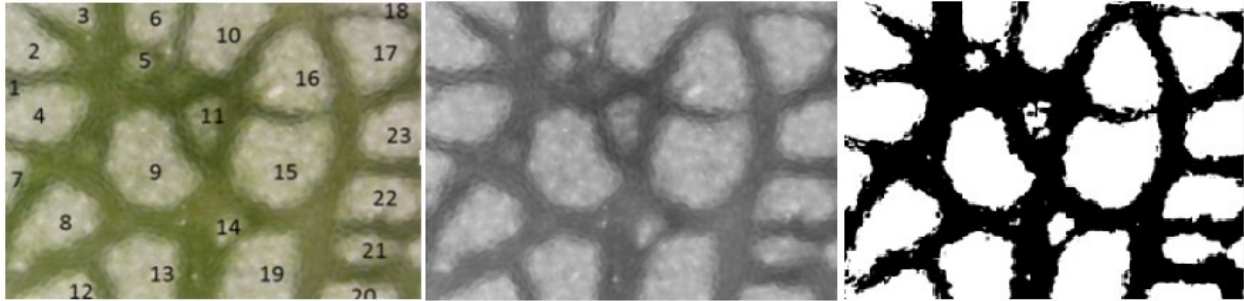


Figure 6. The first image shows the numbering to count vein loops in each image. The subsequent images display turning the original image to grayscale and finally to black and white to count pixels to calculate density.

Punctured vs. unpunctured. I used t-tests to test for the difference in leaf vein density and the number of leaf vein loops between punctured and non-punctured leaves. I average all quadrants in the leaves through Microsoft Excel 365 before using RGui 4.1.2 (R Core Team, 2021) to implement the paired t-test. I used the dplyr package (Wickham 2014) to carry out the t-test.

Quadrants 1 and 4 vs. 2 and 3. I used a paired t-test to test the difference in leaf vein density and the number of leaf vein loops between the center microscope images (2 and 3) with the outer microscope images (1 and 4). I averaged the center and outer quadrants in both the punctured and non-punctured leaves through Microsoft Excel 365 before using RGui 4.1.2 (R Core Team, 2021) to implement the paired t-test. I used the dplyr package to carry out my t-test.

Petiole size vs. leaf vein density. By implementing a multilinear regression, I determined how petiole diameter and surface area affect leaf vein density in punctured leaves. I averaged all of the quadrants in the leaves in Microsoft Excel 365 before using RGui 4.1.2 to carry out a multilinear regression, $LVD \sim \text{petiole diameter} + \text{surface area}$ with β_1 being petiole diameter and β_2 being surface area. I used the car (Fox 2019) package to carry out the multilinear regression.

RESULTS

Punctured leaves vs unpunctured leaves

The leaf vein density of the punctured leaves (Mean = 67,479 pixels, SD = 11,020 pixels) was significantly greater than the leaf vein density of non-punctured leaves (M = 53,622, SD = 8,176), $t(32) = 4.040$, $p = 0.00144$. The range and spread of the leaf vein densities on the punctured leaves were much larger in the punctured leaves than in the control group. There were a higher number of leaf vein loops in the punctured leaves than in the control group. Some of the leaf vein loops of the punctured organisms also exhibited more pronounced and darker minor vein structures compared to the control group, where the minor veins were just beginning to form inside the already defined vein loops.

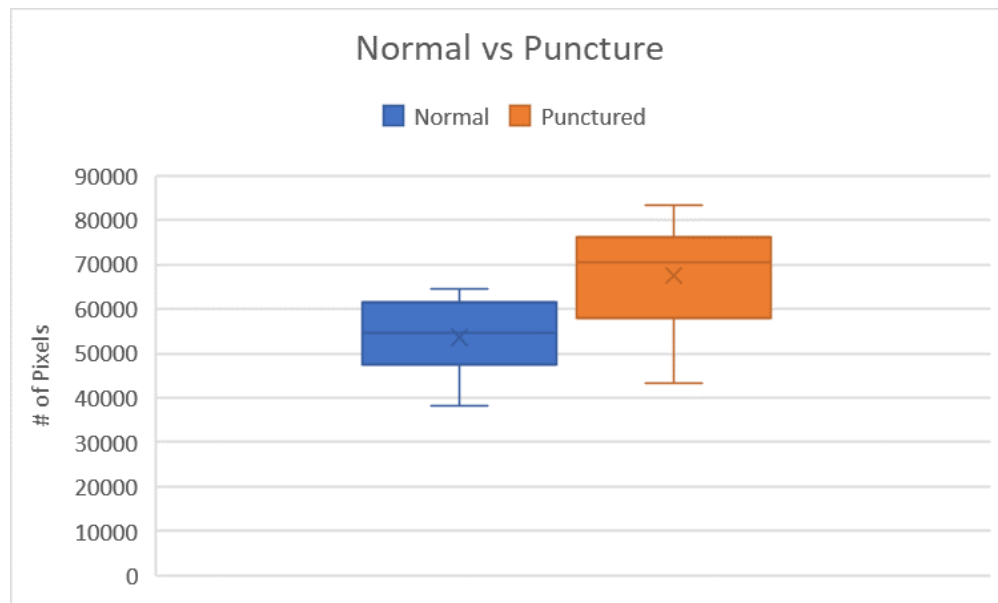


Figure 7. Average leaf vein density measurements comparing samples punctured and without punctures. The control group had a lower median of 54,760 pixels compared to the experimental group, with a median of 70,417 pixels.

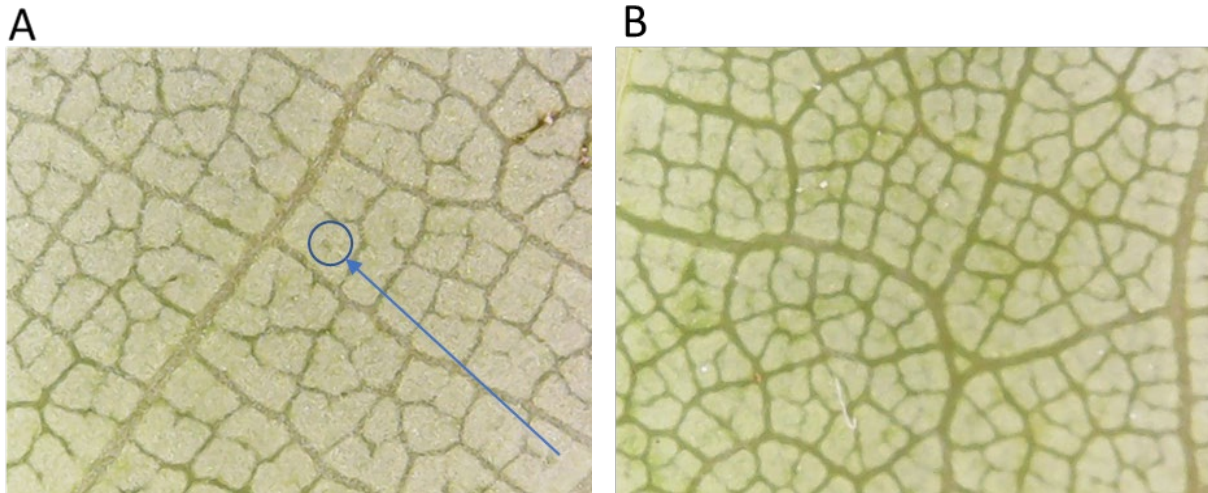


Figure 8. There is a statistically significant difference in leaf vein density between quadrants in punctured leaves, with the median of the far quadrants at 66,772 pixels and the median of the close quadrants at 72,014 pixels.

LVD vs Location

The leaf vein density also differed across the different quadrants in the experimental group. The leaf vein density of the outer quadrants 1 and 4 ($M = 64,728$, $SD = 10540$) was significantly lower than the leaf vein density in the inner quadrants 2 and 3 ($M = 70,230$, $SD = 11969$), $p = 0.000354$. The leaf vein densities in the control group displayed no difference in leaf vein densities with quadrants 1 and 4 ($M = 53,742$, $SD = 8640$) having bearing similar characteristics to quadrant 2 and 3 ($M = 53,501$, $SD = 8388$), $t(16) = 0.0566$, $p = 0.842$. Nevertheless, the experimental group's quadrants 1 and 4 ($M = 64,728$, $SD = 10540$) were significantly different from the control group's quadrants 1 and 4 ($M = 53,742$, $SD = 8640$), $t(16) = 2.280$, $p = 0.00716$. The experimental group's quadrants 2 and 3 ($M = 53,742$, $SD = 8640$) were also significantly different from the control group's quadrants 2 and 3 ($M = 70,230$ $SD = 11969$), $t(16) = 3.237$, $p = 0.000512$.

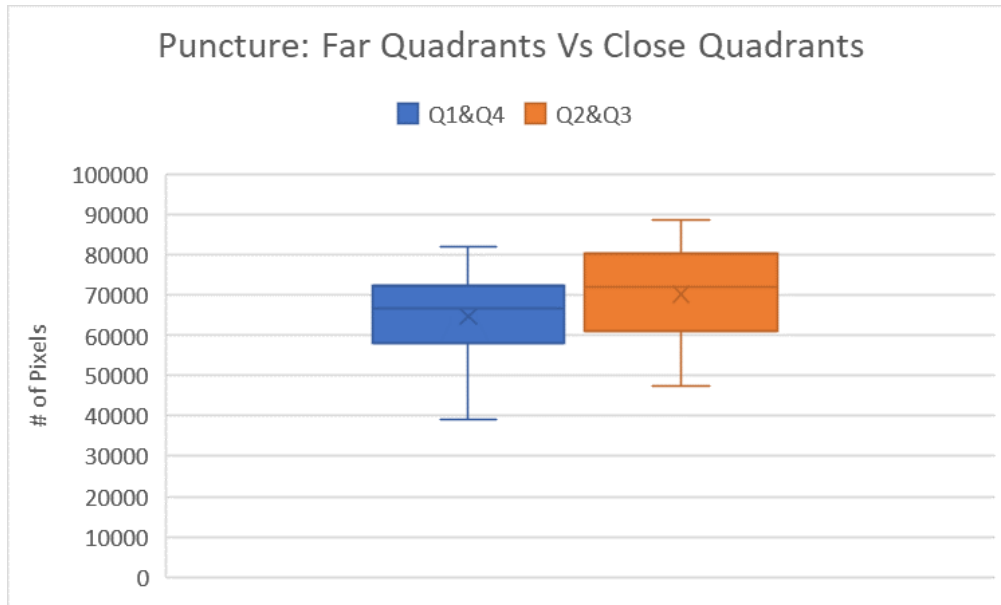


Figure 9. There is a statistically significant difference in leaf vein density between quadrants in punctured leaves with median of the far quadrants at 66,772 pixels and median of the close quadrants at 72,014 pixels.

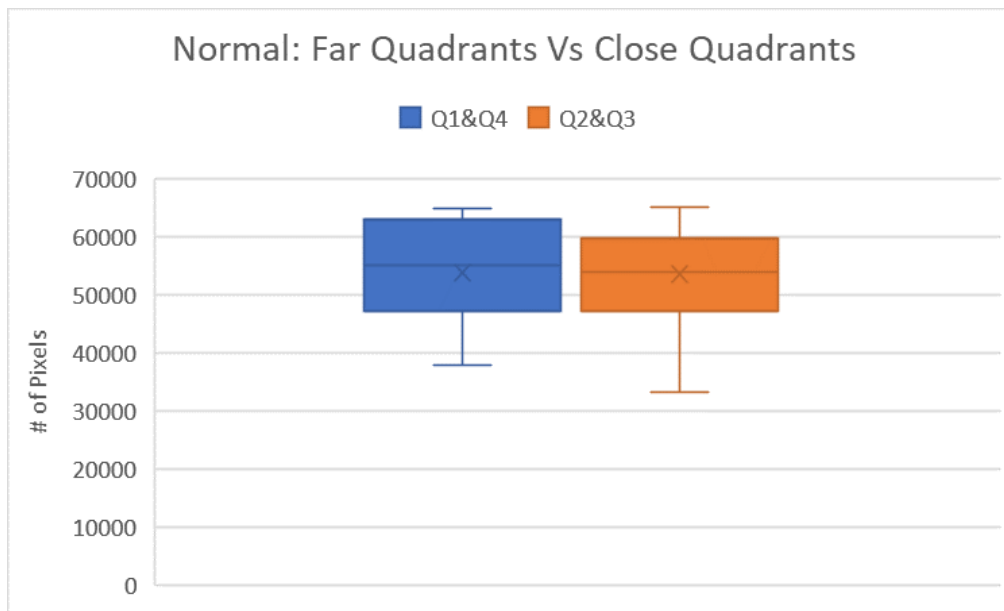


Figure 10. There is no difference in leaf vein density in the control group, with medians at 53,918 pixels for far quadrants and 55,032 pixels for close quadrants.

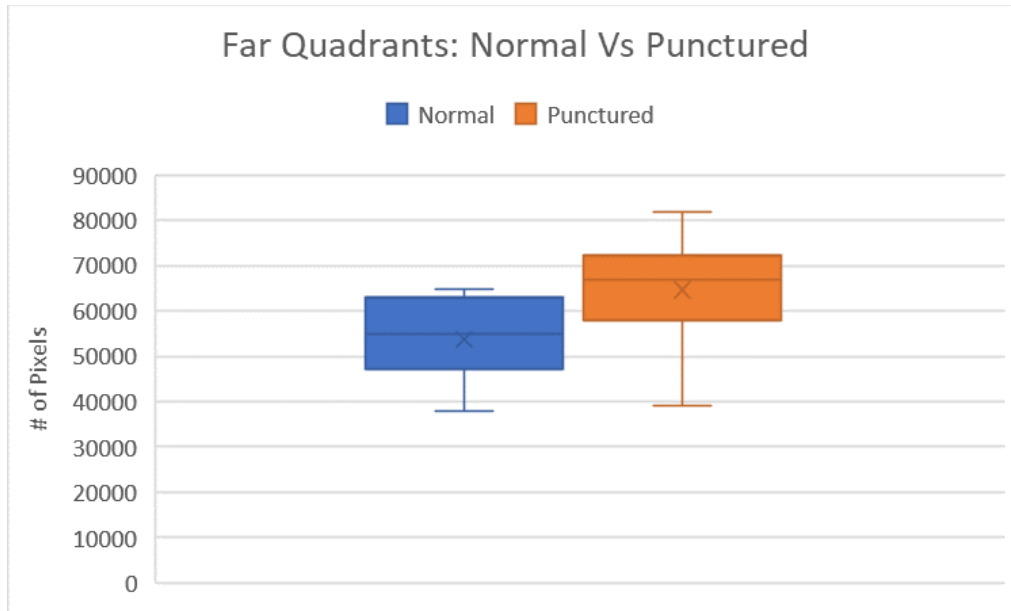


Figure 11. There is a significant difference in leaf vein density between normal and punctured in the far quadrants. The control group in far quadrants has a median of 55,032 pixels and punctured leaves in far quadrants with a median of 66,773 pixels.

LVD vs Petiole Diameter Growth

The leaf vein density varies across the different petiole differences from 0 mm to 0.42 mm, with the highest leaf vein density found at an intermediate petiole diameter of 0.16 mm. Before the puncture wound was inflicted, the petiole diameter did not correlate with the leaf vein density. However, two weeks after the puncture wound was inflicted, the petiole diameter also created a curvy shape, with a leaf vein density being the highest at a certain petiole diameter. Nevertheless, the most noticeable relationship was present when I compared leaf vein density to the difference in petiole diameter before and after the puncture wound. There is also an outlier at -0.33 mm, whose petiole diameter decreased.

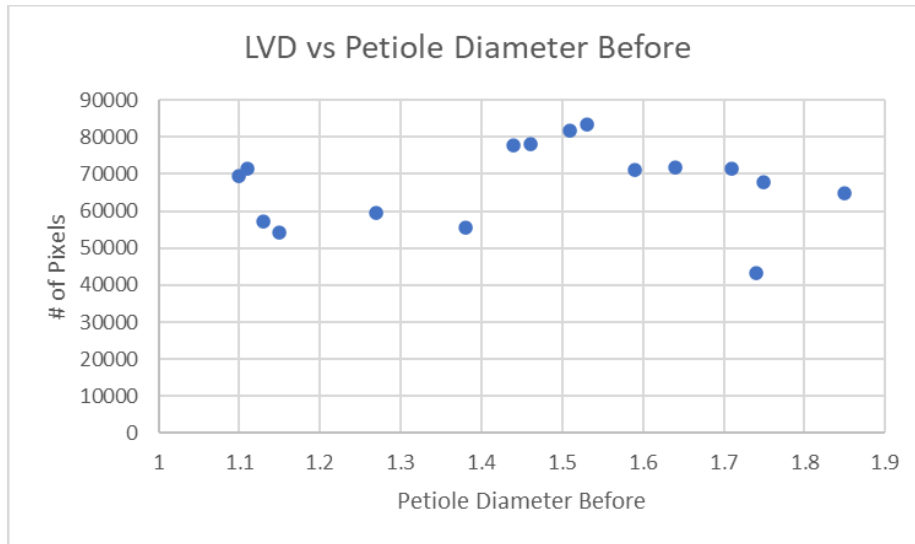


Figure 12. Leaf vein density did not have any interesting relationship with petiole diameter before the puncture.

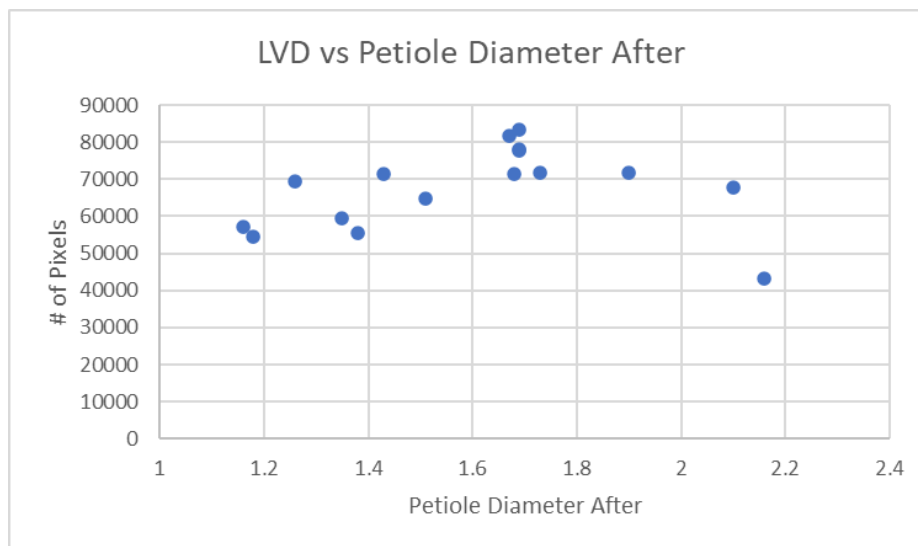


Figure 13. Leaf vein density followed a similar shape to the LVD vs Petiole Diameter Difference, with LVD vs Petiole Diameter Difference accentuating the curve.

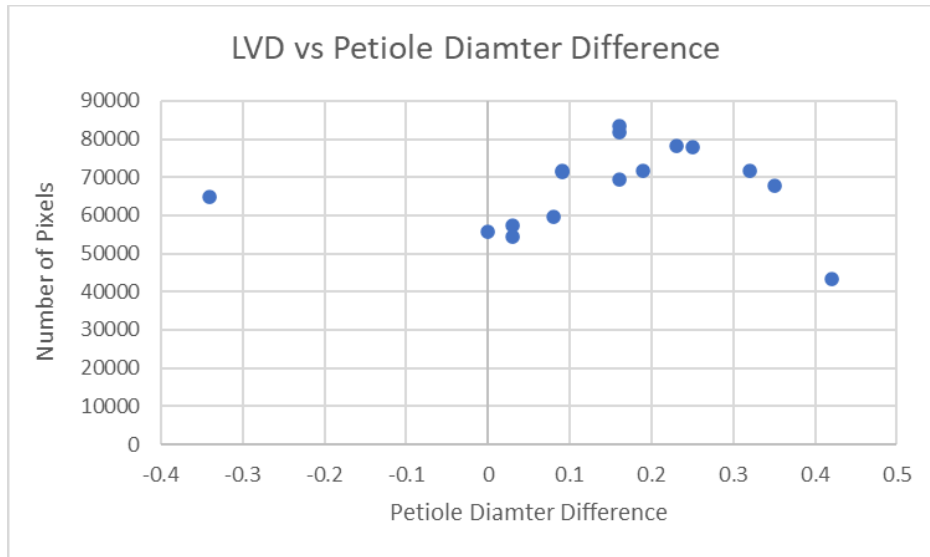


Figure 14. The difference from growth in the petiole diameter results in an arc shape formation in leaf vein density.

LVD vs Surface Area

When we take into consideration surface area in relation to leaf vein density, not much of a relationship can be found between these variables. Similarly, there is no correlation when taking leaf vein density and comparing it to surface area divided by petiole diameter. However, when taking into account surface area and petiole diameter, there is more of a linear correlation with constant slope compared to just comparing leaf vein density and petiole diameter.

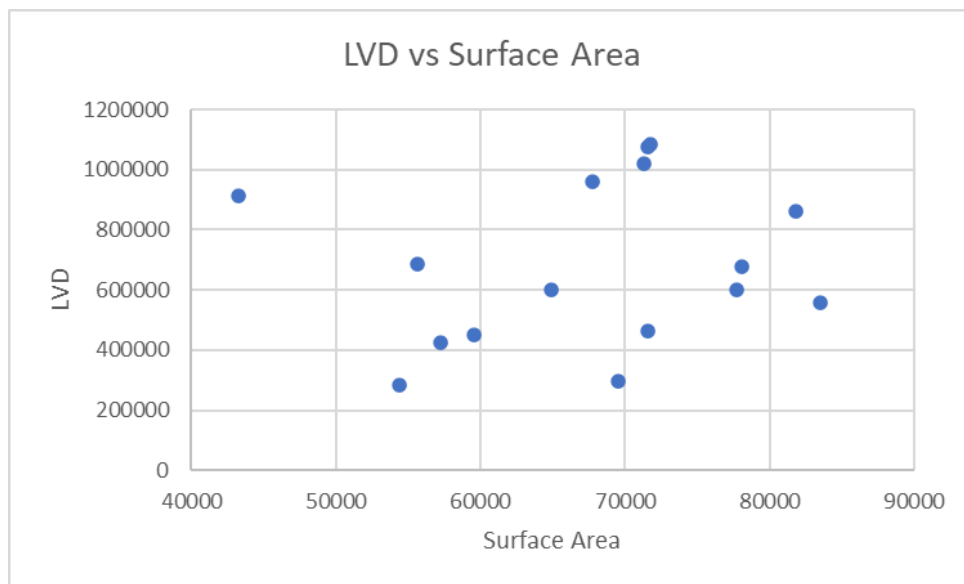


Figure 15. There is no relationship between leaf vein density and surface area.

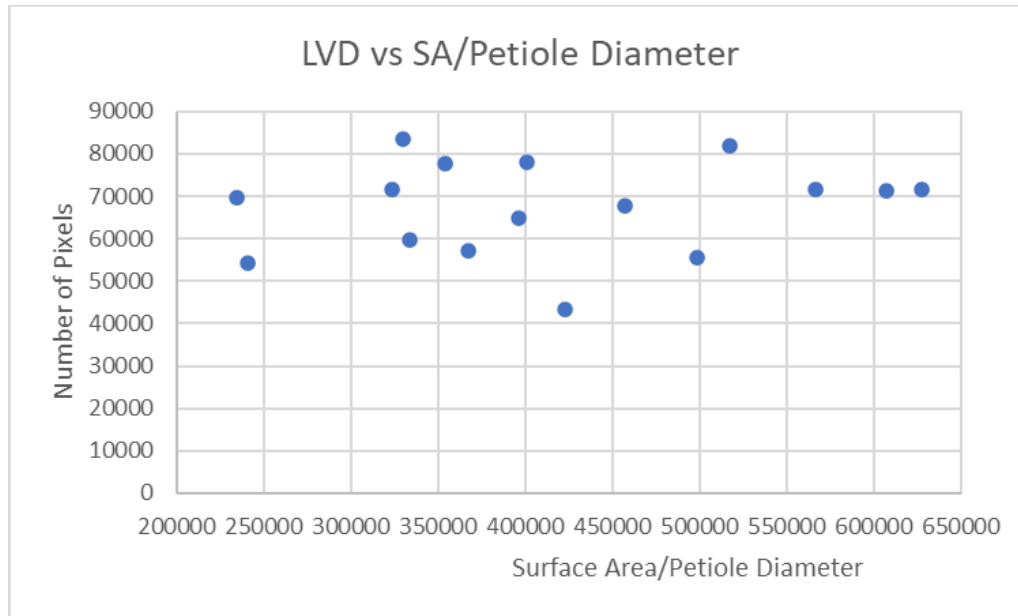


Figure 16. There is no relationship between LVD and surface area/petiole diameter. Graph appears linear though with a constant slope.

DISCUSSION

Discussion introduction

Although climate change has created suboptimal conditions for plants because of the increase in insect herbivory, leaves have developed complex mechanisms to compensate for damages. Leaf venation systems have adapted by creating denser minor vein structures, overcompensating before damages ensue, and abiding by fluid mechanics to create optimal pathways for water transportation to occur. Even after *C. canadensis* sustained a large gape to its central midrib, the experimental leaves were still a lush green with little to no difference from a first glance. However, the results have displayed statistically significant differences between the leaf's veins in *C. canadensis*, suggesting that the leaf compensates at a microscopic level. Most notably, a compelling discussion was drawn from the difference between the normal and punctured trials, the importance of fluid mechanics in leaves, and the association between petiole diameter, surface area, and leaf vein density.

Normal vs punctured trials

Significant differences in leaf vein density between the control and experimental groups were found. These vein density changes are similar to other types of species, such as rainforest canopy angiosperm trees (Lowman 2006). Although we see higher vein densities in damaged angiosperms, this theory is only applied to angiosperms because existing research explains how the high vein densities are not found in any other clade of vascular plants (Boyce et al. 2009). Additionally, the strengthening and thickening of the minor veins is a byproduct of an angiosperm's naturally high vein densities and ability to invest in its veins. This phenomenon can clearly be seen in *C. canadensis*, which was induced to have thicker minor vein concentration when damaged. Most angiosperm veins are popularly experimented on because of their easy-to-see veins, which are theorized to come from their ability to frequently overinvest (de Boer et al. 2016). These higher vein concentrations are hypothesized to be an evolutionary advantage in angiosperms that allow for higher transpiration rates and photosynthesis when damaged.

Another observation I made between the control and punctured trials was the strange incomplete vein formation that primarily existed in the control group. Whereas the punctured leaves had fully formed veins, the non-punctured leaves would often have a fragment of a vein that was not attached to any other vein loops rendering water transportation unused in the segment. However, because these structures were found primarily in the control group, this may also be an evolutionary mechanism developed where the leaves prepare these structures in the face of damage (Wiggins et al. 2016). Hence, when leaves are stressed, the canalization process can transition much more smoothly, filling in veins much faster than without (McAdam and Brodribb 2013). Some researchers also found that auxin has an important role to play in the canalization of the leaf vein loops, as when damaged, the "flux bifurcator" activates in cells with high auxin fluxes—the closed veins—to flow into areas with low auxin fluxes—the incomplete vein formation (Feurgier and Iwasa 2006).

Area of puncture and fluid mechanics

Whereas the quadrants in punctured leaves away from the midrib puncture site also showed a lower leaf vein density than the quadrants right next to the midrib, the control leaves had no

difference between quadrants. Fluid mechanics can explain why when one path is blocked or unable to transport water, the flow rate of other paths must increase their capabilities to compensate (Leopold and Langbein 1960). Nevertheless, the far quadrants still were significantly different from the control group, which indicates that the burden of the puncture must be endured by all of the veins in the leaf, with heavier influence on the veins closer to the puncture site.

Although basic fluid mechanics and findings suggest that higher flow rates lead to thicker veins, wetter conditions should also lead to thicker veins. However, this is not the case as overinvestment in leaf veins occurs in arid habitats (de Boer et al. 2016). Thus, trees in dry climates, such as Utah, will focus on their leaves by investing in those leaf venation systems, whereas trees in arable climates will invest in more leaves. The difference in climate is essential as the amount of water a leaf may have been receiving during the experiment may have drastically different impacts on the leaves (Magendans 1983). Since both the control and experimental groups were done simultaneously, the climate conditions should not explain the differences in leaf vein density. However, they may explain the severity of induced impacts. Nevertheless, because of the unregulated change in the environment, there could be differences among trees in different locations, altitudes, and amounts of rainfall even across the same species *C. Canadensis*.

Petiole diameter, surface area, and leaf vein density

Not much research has linked how petiole diameter plays a role in leaf venation systems when damaged. The highest leaf vein density was found at a petiole diameter growth of 0.16 mm. The peak at 0.16 may be the case as petiole diameter does correlate a little bit with the size of the leaf, the age of the leaf, and the location it is related to the sun. Because of these specific parameters, the leaves that received the highest leaf vein densities might have had the most favorable conditions related to age, size, and location (Weijschedé et al. 2006). Whereas trials outside those parameters may have still received some increase in leaf vein density, those that could still grow 0.16 mm had the most favorable prospects. Thus, the tree would invest in developing its veins as auxin favors the favorable parts of the tree (Mroue et al. 2018). Although this may be a possibility of the curve (Figure 13), another possibility is that the severity of the puncture is directly linked to the growth of the leaf and its venation systems. For the leaves that grew past 0.16 mm, perhaps those leaf systems no longer rely on their major vein as their minor

veins were already used for most of the transport. The explanation may be a combination of both theories as well as others.

A similar consensus across most findings on leaf venation architecture concludes that water potential is the main governing factor in leaf venation systems. De Boer et al. 2016 found that venation architecture in small leaf size—as a result of drought conditions—to be significantly greater in density than larger leaves receiving plentiful amounts of water. Because the size of a leaf plays an important role in its leaf density and should play an important role in its leaf vein structures, the size may be able to also offer some insight into how the leaf relates to the leaf vein density (Salleo et al. 2002). However, the leaf's surface area appeared to have no relationship with leaf vein density than the petiole diameter. Likewise, no relationship was present when both petiole diameter and surface area were compared to leaf vein density. However, Rodriguez et al. 2013 note that morphogenesis in leaves regulates the size of leaves. Leaves are most optimal at a specific size for different species; hence, even with stress such as a puncture wound, the leaf size should not be too contrasting. However, the number of leaves will be drastically lower in the experimental trial because photosynthesis requires adequate water. With less water available in the experimental group, there is no need to make an abundance of leaves.

Limitations and future research

Nevertheless, there are many limitations to this project. As noted earlier, the trials were not done in a lab or greenhouse setting where water, temperature, amount of sunlight, and humidity could change. Another drawback is that using image analysis with a program will assume that the leaf's veins are circular when calculating LVD, which may not be the case. Image analysis also had many parameters that were expected to shift with each leaf. Hence, the transition of leaf vein images from colored to black and white was done by a human, which may have some minor human error. Additionally, there were only 16 experimental trials. Lastly, another challenge is that all leaves can be shaped differently, contain different weights, and have different surface areas, which makes it challenging to find pairings between the control group and the two experimental groups.

In future experiments, I would like to expand this procedure to other species of angiosperms. Suppose there are certain leaves that adapt better to damages to leaves. In that case, it may be possible for genetic modification of certain species to be more resilient when faced with

less desirable conditions. Not only would I like to gather more trials, but I would also like to study the total number of vein loops in a leaf when inflicted with damage. This would require me to examine the entire leaf as a whole rather than just a rectangular microscopic image that could vary due to variability. Additionally, the surface area was only examined during the end of the two growth weeks, but having a before and after could give more insight into what is going on in the venation systems. By closely both surface area and petiole diameter, I would also need to pay attention to the weight of the leaf as different weights indicate the amount of water in the leaf. Lastly, I would like to account for the leaf vein density as three-dimensional in future experiments. This project was measured through photo imaging, which assumes that all veins are perfectly circular, which is not always the case.

Broader implications

In conclusion, leaves are resilient to punctures by developing complex minor vein systems to compensate for the damage to their major veins. Venation systems are complex to allow for nutrient transport and delivery flexibility, which explains why many leaves can still stay lush green even after being decimated by insect herbivory. Many of the complex mechanisms of leaf venation systems display the evolutionary mechanisms that leaves have to deal with environmental stressors such as chewing insects. Although the rise of temperatures leads to more favorable conditions for insect herbivory such as locust swarms, angiosperms will be able to adapt because of natural defense mechanisms in their leaf venation systems. Although agriculture and gardens may suffer in their productivity and quantity, plants will continue to transform.

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