

# **FROM BIRTH TO DEATH: UNRAVELING THE MYSTERIES OF DECIDUOUS FOLIAGE**

by

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*To my mother who provided endless support for my work and education.*

*To my father who always encouraged learning the next thing.*

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# TABLE OF CONTENTS

LIST OF TABLES .....	10
LIST OF FIGURES .....	11
ABSTRACT .....	15
CHAPTER 1. GENERAL INTRODUCTION .....	17
1.1 Water loss in expanding leaves .....	18
1.2 Stomatal responses to drought in deciduous anisohydric species .....	19
1.3 The role of ABA in leaf senescence .....	20
1.4 How leaves survive freezing .....	21
1.5 Climate change changing phenology .....	22
1.6 Structure of this thesis .....	23
1.7 References .....	24
CHAPTER 2. A PERMEABLE CUTICLE, NOT OPEN STOMATA, IS THE PRIMARY SOURCE OF WATER LOSS FROM EXPANDING LEAVES .....	33
2.1 Abstract .....	33
2.2 Introduction .....	33
2.3 Methods .....	36
2.3.1 Plant material .....	36
2.3.2 Determining cuticular and stomatal conductance by leaf gas exchange .....	36
2.3.3 Quantifying foliage abscisic acid levels .....	37
2.3.4 Anatomy .....	38
2.3.5 Leaf water potential .....	39
2.4 Results .....	39
2.5 Discussion .....	48
2.6 Acknowledgments .....	50
2.7 References .....	51
CHAPTER 3. ABSISIC ACID DRIVEN STOMATAL CLOSURE DURING DROUGHT IN ANISOHYDRIC <i>FAGUS SYLVATICA</i> .....	56
3.1 Abstract .....	56
3.2 Introduction .....	56

3.3	Methods.....	59
3.3.1	Plant material .....	59
3.3.2	Data collection .....	59
3.3.3	Data analysis .....	61
3.4	Results.....	62
3.5	Discussion .....	67
3.6	Acknowledgments.....	70
3.7	References.....	71
CHAPTER 4. ABSCISIC ACID CAN AUGMENT, BUT IS NOT ESSENTIAL FOR, AUTUMNAL LEAF SENESCENCE .....		78
4.1	Abstract .....	78
4.2	Introduction.....	78
4.3	Methods.....	84
4.3.1	Plant Material.....	84
4.3.2	Girdling experiment.....	85
4.3.3	Measurements .....	85
4.3.4	Data analysis .....	87
4.4	Results.....	90
4.4.1	Variation in the onset and duration of senescence does not coincide with a change in ABA levels.....	90
4.4.2	Senescence onset timing and rate are enhanced by high ABA levels induced by girdling.....	90
4.4.3	Senescence is not driven by a loss of hydraulic conductance .....	91
4.5	Discussion .....	95
4.5.1	ABA can enhance deciduous leaf senescence but does not increase at the onset of senescence.....	95
4.5.2	Leaf dehydration and loss of conductance does not drive annual leaf senescence ...	96
4.5.3	Stomata close during senescence and may drive autumnal leaf senescence .....	97
4.5.4	Conclusion .....	97
4.6	Acknowledgments.....	98
4.7	References.....	98

CHAPTER 5. SPATIAL AND TEMPORAL FREEZING DYNAMICS OF LEAVES REVEALED BY TIME-LAPSE IMAGING.....	105
5.1 Abstract.....	105
5.2 Introduction.....	105
5.3 Methods.....	108
5.3.1 Plant material.....	108
5.3.2 Visualizing leaf freezing.....	108
5.3.3 Determination of the freezing point of leaf, stem, and xylem sap.....	109
5.3.4 The effect of freezing speed on recovery .....	110
5.3.5 Assessing maximum photosynthetic rates of leaves exposed to air and buried under snow under repeated freezing and thawing. ....	110
5.3.6 Data analyses and statistics.....	111
5.4 Results.....	113
5.4.1 High resolution evaluation of leaf freezing using timelapse imaging .....	113
5.4.2 Effect of freezing speed on photosynthetic and water potential recover .....	113
5.4.3 Insulation by snow can protect leaves from severe freezing events.....	116
5.5 Discussion.....	117
5.6 Acknowledgements.....	119
5.7 References.....	120
5.8 Supplemental material .....	126
CHAPTER 6. HIGH PHENOLOGICAL DIVERSITY BLURS THE PREDICTIONS OF FUTURE FOREST RESPONSES TO CLIMATE CHANGE .....	128
6.1 Abstract.....	128
6.2 Main text .....	128
6.3 Acknowledgments.....	132
6.4 References.....	132
CHAPTER 7. CONCLUSIONS AND FUTURE WORK.....	134
7.1 Water, stomata, and expanding leaves.....	135
7.2 Drought, heat, and ABA .....	136
7.3 Senescence, abscission, and leaf death .....	137
7.4 Freezing.....	140



7.5	Tree phenology in a changing world .....	141
7.6	Conclusions.....	142
7.7	References.....	142

## LIST OF TABLES

Table 3-1. The collection dates as days since winter solstice (DAWS) and day of year (DOY). Accompanying each date is the mean and standard errors (n=3) of leaf water potential, foliage ABA level, leaf chlorophyll <i>a</i> content, stomatal conductance, photosynthetic rate and water use efficiency (WUE).....	62
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## LIST OF FIGURES

Figure 2-1. (A) The percentage of transpired water lost through stomata as *Quercus rubra* leaves expand. The insert depicts the absolute rates of leaf conductance measured in the same leaves. Generalized additive model curves and 95% confidence intervals are represented by solid and dashed black lines, respectively. Each point represents a single leaf. Letters on the chart depict the leaf from which representative images B and C were taken. (B) Cross sections through the epidermis of a *Q. rubra* leaf 6 days after emerging, and (C) 21 days after emerging, with cuticles stained with Sudan IV (scale bars = 10µm). ..... 40

Figure 2-2. Mean leaf area of *Quercus rubra* leaves from emergence (day 0) to 23 days after leaf emergence (n = 8 leaves, ± SD). Dashed lines depict standard deviation. .... 41

Figure 2-3. Foliage abscisic acid (ABA) level in expanding *Quercus rubra* leaves. ABA levels are expressed in terms of dry weight. A single exponential decay 3 parameter model (ABA level DW =  $0.3822 + 24.2829 \times e^{-0.1340 \times \text{Leaf age}}$ ) (solid line) with 95% confidence interval (dashed lines) is depicted (P = <0.0001, R<sup>2</sup> = 0.8493). The insert shows ABA levels in terms of fresh weight (FW). A single exponential decay 3 parameter models (ABA level FW =  $-0.0982 + 3.6244 \times e^{-0.0737 \times \text{Leaf age}}$ ) (solid line) and 95% confidence interval (dashed line) are depicted (P = <0.0001, R<sup>2</sup> = 0.7912). ..... 42

Figure 2-4. (A) Mean stomatal density (n = 5 fields of view per leaf taken from the center of the leaf, ±SE) of expanding *Quercus rubra* leaves. Each point represents a single leaf. Letters on the chart depict the leaf from which representative images B, C and D were taken. Generalized additive model curves and 95% confidence intervals are represented by solid and dashed black line respectively. The insert represents the total number of stomata per leaf of expanding *Quercus rubra* leaves (solid line) flanked by the 95% confidence interval (dashed line). (B) An image of the abaxial surface of a *Quercus rubra* leaf three days after emergence with visible trichomes (scale bar = 80µm). (C) An image of the abaxial surface of a *Quercus rubra* leaf thirteen days after emergence with (scale bar = 80µm). (D) An image of the abaxial surface of a *Quercus rubra* leaf twenty-seven days after emergence (scale bar = 80µm). ..... 43

Figure 2-5. (A) Mean percentage of stomata with an aperture (n = 5 fields of view per leaf taken from the center of the leaf, ± SE) in expanding leaves of *Quercus rubra*. Each point represents a single leaf. A logistic 3 parameter sigmoidal curve (solid line) and 95% confidence interval (dashed line) is shown (P = <0.0001, R<sup>2</sup> = 0.7178). (B & C) Representative images of *Quercus rubra* stomata (B) without an aperture and (C) with an aperture captured on the same leaf 10 days after emergence (scale bar = 10 µm). ..... 45

Figure 2-6. Mean stomatal density on the abaxial surface (n = 5 fields of view from the same leaf taken from the center of the leaf, ± SE) in expanding *Arabidopsis thaliana Col-0* leaves. Each point represents a single leaf. A Rational, 2 Parameter II curve (solid line) and 95% confidence interval (dashed line) is shown (P = <0.0015, R<sup>2</sup> = 0.8870). ..... 46

Figure 2-7. (A) Mean percentage of stomata that have formed an aperture on the abaxial surface ( $n = 5$  fields of view per leaf taken from the center of the leaf,  $\pm$  SE) in young expanding leaves of *Arabidopsis thaliana Col-0*. Each point represents a single leaf. A Rational, 3 Parameter II (solid line) and 95% confidence interval (dashed line) is shown ( $P = <0.0050$ ,  $R^2 = 0.9295$ ). (B) Image of an *A. thaliana Col-0* stoma without an aperture on a leaf that was 29.04 mm<sup>2</sup>, approximately 6 days after emergence (Scale bar = 5 $\mu$ m). (C) Image of an *A. thaliana Col-0* stoma with an aperture on with the same leaf imaged in B (Scale bar = 5 $\mu$ m)..... 47

Figure 3-1. (A) Mean leaf ABA content, (B) stomatal conductance, (C) leaf water potential, (D) leaf chlorophyll *a* content through a natural drought in an individual of *Fagus sylvatica f. purpurea* in West Lafayette, Indiana, USA. Each black point represents an average of 3 sun exposed leaves measured at midday and are accompanied with standard error bars. The two white and one red point on 271 days after winter solstice show the individual values of leaf ABA content, stomatal conductance, leaf water potential for each leaf collected on that day with the trend line passing through the average of those points. The insert in A depicts the rain fall during the study period with the yellow area indicating the abnormally dry period (D0). The insert in (B) depicts water use efficiency through the drought. One-way ANOVAs were performed on all time course data and the F-statistic and P value are displayed, Tukey's post hoc tests were performed, and significant differences are denoted by different letters..... 64

Figure 3-2. (A) The relationship between leaf ABA content and stomatal conductance and (B) leaf water potential and leaf ABA content for leaves sampled through a natural drought in West Lafayette, Indiana, USA in 2021. Each point represents one sun leaf collected during midday from an individual *Fagus sylvatica f. purpurea*. Exponential decay regressions are fitted to the data with dashed lines representing 95% confidence intervals. The vertical solid line in B depicts the mean turgor loss point of 6 individual sun leaves from the same individual with the dashed vertical lines showing the standard error of the turgor loss point. Insert in (A) shows the linear relationship between stomatal conductance and leaf water potential. (C) Pressure-volume curve for leaves of *Fagus sylvatica f. purpurea*. Each point represents one measurement of one of the 6 leaves with each leaf being measured 9 times during the dry down. The vertical solid line shows the mean turgor loss point of 6 individual sun leaves from the same individual with the dashed vertical lines showing the standard error of the turgor loss point. .... 66

Figure 4-1. Foliage chlorophyll *a* content from mid-September 2020 in girdled (grey) and ungirdled (black) branches of *Phellodendron amurense* (A), *Lonicera  $\times$  purpusii* (B), *Ginkgo biloba* (C) and *Quercus falcata* (D). The solid lines depict a general additive model with  $R^2$  values shown, while the dashed lines demarcate standard error. Each point represents a single leaf. .... 83

Figure 4-2. Foliage ABA content from mid-September 2020 in girdled (grey) and ungirdled (black) branches of *Phellodendron amurense* (A), *Lonicera  $\times$  purpusii* (B), *Ginkgo biloba* (C) and *Quercus falcata* (D). The solid lines depict a general additive model with  $R^2$  values shown, while the dashed lines demarcate standard error. Each point represents a single leaf. .... 84

Figure 4-3. Mean and standard error of foliage abscisic acid levels in girdled (white) and ungirdled (black) branches from 3-20 days after girdling ( $n=15$ ). \* denotes a significant difference in means ( $P < 0.05$ , T-Test)..... 88

Figure 4-4. Leaf water potential from mid-September 2020 in girdled (grey) and intact (black) branches of *Phellodendron Amurense* (A), *Lonicera × purpusii* (B), *Ginkgo biloba* (C) and *Quercus falcata* (D). . The solid lines depict a general additive model (GAM) with  $R^2$  values shown, while the dashed lines demarcate standard error. Each point represents a single leaf. .... 89

Figure 4-5. The relationship between  $CO_2$  assimilation rate and stomatal conductance measured in leaves from all species from late summer until the end of the autumn. The solid line fits an Exponential Rise to Maximum, Single, 2 Parameter (Assimilation rate= $21.519 * (1 - (-4.6816 * \text{stomatal conductance}))$ ). The dashed lines represent the 95% confidence interval of the fit curve. Each point represents one leaf measured. Squares represent *Phellodendron amurense*, hexagons *Lonicera x purpusii*, triangles *Ginkgo biloba* and diamonds *Quercus falcata*. and. Black symbols indicate data collected in 2018, white 2019, and grey in 2020. .... 93

Figure 4-6. The relationship between the percentage of maximum chlorophyll *a* content in leaves and percentage of maximum  $CO_2$  assimilation rate in all species from late summer until the end of the autumn. The solid line fits an linear relationship, (Percent maximum assimilation rate= $5.7428 + 0.7559 * \text{Percent maximum chlorophyll } a \text{ content}$ ). The dashed lines represent the 95% confidence interval of the fit curve. Each point represents one leaf measured. Squares represent *Phellodendron amurense*, hexagons *Lonicera x purpusii*, triangles *Ginkgo biloba* and diamonds *Quercus falcata*. Black symbols indicate data collected in 2018, white 2019, and grey in 2020. .... 94

Figure 5-1. A. Shows a 450 by 450-pixel section of the first raw image of the image stack taken during a natural in-situ freeze thaw cycle of *Lonicera × purpusii*. The white scale bar indicates 2.5cm B. The 450 by 450-pixel section broken down into 45 by 45-pixel sections with each section colored based on the leaf temperature at which the mean pixel brightness increased by 10% from the initial value. With veins outlined in black. C. The same leaf section showing the leaf thawing temperature based on a decrease of 10% mean pixel brightness from maximum pixel brightness. D. Show a 90 by 90-pixel subsection of the first image from the raw image stack. The white scale bar is 0.5cm E. The 90 by 90-pixel section broken down into 9 by 9-pixel sections with each section colored based on the leaf temperature at which the mean pixel brightness increased by 10% from the initial value. With veins outlined in black. F. The same leaf section showing the leaf thawing temperature based on a decrease of 10% mean pixel brightness from maximum pixel brightness. G. The difference in mean freezing temperature between sections of the 90 by 90-pixel section that contain the veins and sections that contain the mesophyll surrounded by vein. The ‘\*’ indicates that they are significantly different based on a two-way student-T test. H. The difference in mean freezing temperature between sections of the 450 by 450-pixel section that contain the midrib, mesophyll adjacent to the midrib, and mesophyll and minor veins farther from the midrib. The letters indicates that they are significantly different based on a one-way ANOVA and a Tukey's HSD post hoc test ( $p < 0.05$ ). I. The time course during the night when freezing was measured out of relative pixel brightness of a 9 by 9-pixel section containing mostly vein (black) or areola (gray) with the leaf temperature (red). A horizontal line demarcated 0°C. The insert depicts the area the brightness was measured in with the black pixel being the vein and the gray pixel mesophyll. 112

Figure 5-2. A. The percent of leaf pixels that have initiated freezing (black) over the course of the night of DOY 28 and the raw leaf temperature on the same night (gray). B. Freezing exotherms of *ex situ* leaves frozen at  $-1^{\circ}\text{C min}^{-1}$  (gray) and  $-10^{\circ}\text{C min}^{-1}$  (black) vs the time in seconds since the leaves reached  $0^{\circ}\text{C}$ . C. Freezing temperatures of leaf frozen at  $-0.01^{\circ}\text{C min}^{-1}$ ,  $-1^{\circ}\text{C min}^{-1}$ , and  $-10^{\circ}\text{C min}^{-1}$  with standard errors. Letters denote a significantly different mean based on a one-way ANOVA and a Tukey's HSD post hoc test ( $p < 0.05$ ). D. Photosynthetic recovery of leaves frozen at  $-0.01^{\circ}\text{C min}^{-1}$ ,  $-1^{\circ}\text{C min}^{-1}$ , and  $-10^{\circ}\text{C min}^{-1}$  with standard errors. Letters denote a significantly different mean based on a one-way ANOVA and a Tukey's HSD post hoc test ( $p < 0.05$ ). E. Change in leaf water potential of leaves frozen at  $-0.01^{\circ}\text{C min}^{-1}$ ,  $-1^{\circ}\text{C min}^{-1}$ , and  $-10^{\circ}\text{C min}^{-1}$  with standard errors. Letters denote a significantly different mean based on a one-way ANOVA and a Tukey's HSD post hoc test ( $p < 0.05$ )..... 115

Figure 5-3. The mean maximum assimilation rate of leaves measured at  $22^{\circ}\text{C}$  that had been exposed to air during the snow storm and cold period (Black points), the white point represents the mean maximum assimilation rate measured on leaves from a branch that was buried under snow from the onset of the snowfall until the day of measuring ( $n=3$ ,  $\pm\text{SE}$ ). The gray line depicts air temperature. The '\*' denotes a significant difference between the maximum assimilation rates of the leaves exposed to air and buried under snow DOY 54..... 116

Figure 6-1. Considerable diversity in the phenological response to warmer climates exists across canopy dominant, temperate deciduous forest species. (A) In a common latitude experiment in which phenology was tracked across a year in clones of three deciduous tree species grown at sites equidistant from the equator in West Lafayette, Indiana, USA (red) and Hobart, Tasmania, Australia (blue) a wide diversity of phenological responses were observed. (B) These sites have similar daylength during the growing season (dotted lines), yet daily mean temperature differs dramatically, with temperature data from both West Lafayette (red) and Hobart (blue) fitted with a general additive model and standard error (dashed line) for the growing season in the years of data collection (West Lafayette in 2021 and Hobart (2022-2023)), the vertical line denotes the summer solstice, while the horizontal line marks  $0^{\circ}\text{C}$ . Mean percent of maximum leaf chlorophyll *a* content ( $n=3$ ,  $\pm\text{SE}$ ) for clones of (C) *Quercus robur* 'Fastigiata', (D) *Platanus x acerifolia* and (E) *Fagus sylvatica* f. *purpurea* grown in West Lafayette (red) and Hobart (blue). The first points represent the day of bud burst and the final point the day of complete leaf loss, the vertical line denotes the summer solstice. All dates are relative to the winter solstice at each site. A representative leaf of each species is shown (scale bar = 2cm). ..... 131

## ABSTRACT

Deciduous leaf habits have evolved multiple times across many lineages in response to stresses like drought, cold, or darkness. This short, seasonal leaf lifespan allows trees to invest in photosynthesis during prime conditions and retreat to dormancy to survive less favorable conditions. The consequence of short leaf lifespan is that trees must perform an entire year's carbon capture into 6-8 months. This leads to leaves that are cheaper to produce than longer lived evergreen counterparts. As soon as challenging conditions have passed the leaves of deciduous trees expand rapidly; and this expansion has huge impacts on local ecosystems. Other plants like spring ephemerals have evolved to complete the majority of their life cycle before the upper canopy closes off. During the summer, deciduous leaves gather huge amounts of carbon for the trees to survive their dormancy. Finally, as the trees prepare to enter dormancy, nutrients are withdrawn from leaves as the chlorophyll is metabolized, causing them to transition from bright green to shades of red and yellow. In addition to other plants, people find the annual process of renewal on bud burst and tragic decline during senescence fascinating and culturally important. The aim of my thesis is to expand our understanding of winter deciduous leaves through every major stage of development, as well as investigating how this process may shift due to climate change.

During leaf expansion, I tested the hypothesis that young leaves have open stomata which are responsible for most of the plant's water loss during expansion, as had been observed in the annual rosette herbaceous angiosperm *Arabidopsis thaliana*. I found that in the deciduous species *Quercus rubra*, stomata do not begin to develop until after leaves have emerged from the bud and that stomatal pores are closed, covered in cuticle, until the leaf is almost fully expanded, with most water being lost through the cuticle.

Once leaves are fully expanded, drought can severely limit the amount of carbon trees are able to sequester during their prime photosynthetic months. It has been hypothesized that some trees, classed as anisohydric, do not use abscisic acid (ABA) to close stomata during drought, rather that stomata are closed passively by lower water status. I measured ABA, stomatal conductance, and water potential in the anisohydric winter deciduous species *Fagus sylvatica* and found that they do produce ABA during drought and that this change in ABA corresponds to declines in stomatal conductance and declining water potentials.

ABA, along with ethylene, has long been thought to play an important role in leaf senescence. Most work in this area has been done on annual herbaceous species including some crops. I set out to test if ABA may also be driving the annual leaf senescence in temperate deciduous species. I observed that none of the four species examined produced higher levels of ABA during leaf senescence, but that if leaf ABA content was artificially increased, it would lead to increased senescence rates.

Plants that do not senesce and shed leaves during the autumn must be able to survive freezing temperatures and repeated freeze-thaw cycles. Some brevideciduous species are able to survive to a lower temperature than most winter deciduous species, but do still lose leaves late in winter. I investigated the thermal limit of leaf survival in a brevideciduous species of *Lonicera*. To do this I developed a new and simple means of observing the temporal and spatial dynamics of freezing and thawing point of leaf tissues *in situ* using time-lapse photography. The speed of freezing was found to be a major determinant of leaf survival during winter in this species, as well as the minimum temperature experienced by leaves.

Climate change has already started disrupting the usual cycles of leaf out and leaf fall in temperate regions of the world. It has been proposed that, opposite to what had been thought for decades, warmer temperatures might shorten deciduous forest growing seasons, or not change them. I set out to test how warmer seasons might impact leaf lifespan in deciduous species by conducting a common latitude experiment across hemispheres in which leaf lifespan was tracked. I found that there is considerable diversity in the effect that changes in temperature have on leaf lifespan and that some trees may gain significant seasonal photosynthetic advantages over other species that coexist in deciduous forests. These changes have not been incorporated into models of future forest responses to changing climates.

The results of this thesis have increased our understanding of deciduous leaves at each major stage of development. Some results have challenged assumptions made from extending discoveries made in annual herbaceous model systems. This thesis has expanded not only our collective knowledge and technical toolkit but also spawned new and important questions about deciduous plants that need answering.



## CHAPTER 1. GENERAL INTRODUCTION

Leaf lifespan in temperate deciduous forests is an important but often mysterious trait that has a strong impact on the phenology of local ecosystems, cultural practices of human communities, and is a major influence on the annual global carbon cycle (Knorr 2000; Richardson *et al.* 2010, 2013; Mittermeier, Roll, Matthews & Grenyer 2019). Temperate forests assimilate around 11 metric tons of carbon per hectare annually (Wofsy *et al.* 1993). This carbon is absorbed primarily in the spring and summer (Bassow & Bazzaz 1998). The phenology of these trees is culturally important to many around the globe (Mittermeier *et al.* 2019). With spring leaf out and flowering bringing a sense of rebirth and new starts to those in the higher latitudes, links have been made to human health, especially for humans still connected to nature in these environments (Pratiwi, Xiang & Furuya 2019; Mittermeier *et al.* 2019). Likewise, senescence and abscission every autumn dazzle humans with vivid colors, often reminding people of how fleeting and ephemeral our time on Earth can be (Mittermeier *et al.* 2019; Jo, Ikei & Miyazaki 2022). Despite how captivating these processes are for humans, the underlying physiological mechanics that drive deciduous tree phenology are poorly understood and hotly debated (Gan & Amasino 1997; Richardson *et al.* 2012; Liu *et al.* 2020). Much of what we know about this process are inferred from work done on annual herbs, likely due to the difficulty of working on large long-lived species without considerable genetic resources available (Fields & Johnston 2005; Lim, Kim & Gil Nam 2007; Pantin *et al.* 2013). The evolutionary origins of the deciduous habit are debated, but it is likely deciduousness has evolved at least 20 times (Wolfe & Upchurch 1986; Wolfe 1987; Tiffney & Manchester 2001; Edwards *et al.* 2017). Popular evolutionary theories suggest that plants evolved this unique leaf habit to better survive at high latitudes due the extremely short days winter (Wolfe & Upchurch 1986; Wolfe 1987). It has also been suggested that deciduousness evolved first as a response to drought near the tropics and was repurposed for survival in the water-limited cold winters of high latitudes (Axelrod 1966). Others have proposed that deciduousness evolved as a direct response to a cooling planet during the Cenozoic era, that allowed plants to survive and spread farther north (Zanne *et al.* 2014). Many angiosperm and gymnosperm lineages likely evolved a deciduous leaf habit in the Late Cretaceous (100-70 million years ago) (Wolfe 1987) while groups like *Viburnum* may have evolved the habit much more recently in the Late Miocene (10-4 million years ago) (Utescher & Mosbrugger 2007; Pound *et al.* 2011). With global climate

change rapidly warming the atmosphere the deciduous growth habit that allows for survival in cold temperatures may become selected against (Menzel & Fabian 1999; Piao, Friedlingstein, Ciais, Viovy & Demarty 2007; Gunderson *et al.* 2012; Godbold *et al.* 2014; Xu, Riley, Koven, Jia & Zhang 2020). Due to climate change temperate forests are under new existential, threats like drought events increasing in frequency and severity (Anderegg *et al.* 2015; Cook, Mankin & Anchukaitis 2018; Frei *et al.* 2022), as well as the increasing risk to expanding leaves of late spring frost and higher insect and fungal disease loads (Weed, Ayres & Hicke 2013; Anderegg *et al.* 2015; Lamichhane 2021). To better understand how deciduous trees will respond to climate change an integrated approach investigating how these leaves develop, function, and die seasonally is critical. In my thesis I set out to better elucidate each of these key stages of the leaf lifespan from investigating water loss in developing leaves to the hormonal drivers of leaf senescence, as well as the temperature thresholds that mark the end of leaf life in brevidciduous shrubs. Lastly, I set out to better understand how leaf lifespan in temperate forests will be affected by our warming world.

## **1.1 Water loss in expanding leaves**

Water loss in levels is primarily controlled by two barriers, the stomata and the cuticle (Körner 1994). Dynamic stomata which open and close in response to a host of stimuli including light, temperature and humidity expressed as vapor pressure deficit (VPD), and plant water status (Brodribb, Sussmilch & McAdam 2020); are responsible for more than 99% of a leaf water loss when open (Körner 1994; Duursma *et al.* 2019). This dynamic system of gated pores is what allows plant membranes to encounter atmospheric CO<sub>2</sub> which is fixed during photosynthesis (Raven 1984; Brodribb *et al.* 2020). The dynamic action of stomata also allows plants to regulate their water status by closing during times of stress to reduce the risk of damage and death due to drought (Brodribb *et al.* 2020). The waxy cuticle layer that spreads across almost the entire leaf surface is a passive barrier that prevents water loss and once the stomata are shut, due to its much larger area cuticular water loss and incompletely closed stomata can become responsible for up to 94% of leaf water loss, albeit at a rate 100 times lower than when stomata are open (Sargent 1976; Šantrůček, Šimánová, Karbulková, Šimková & Schreiber 2004; Brodribb, McAdam, Jordan & Martins 2014; Duursma *et al.* 2019). Cuticles likely evolved very early after the transition to land, and the combination of stomata and cuticle is likely a major factor in plants being able to

successfully leave the soil boundary layer, grow taller, and take over the terrestrial planet (Raven 1984).

Leaf expansion is a critical process for almost all land plants, and in deciduous forests it is what allows for the rapid and complete replacement of the entire canopy every spring, but these young leaves are very sensitive abiotic stress, particularly drought (Hsiao & Xu 2000). The careful coordination of cell development and expansion, and all while maintaining leaf water status is essential for the rapid development of a canopy in deciduous trees (Shackel, Matthewes & Morrison 1987; Hsiao & Xu 2000; Liu, Jensen & Andersen 2003; Siebrecht, Herdel, Schurr & Tischner 2003). High turgor pressure is needed to force cell walls to expand, this is in tension with the need to have a thin cuticle that will allow for proper leaf growth, but this lack of a developed cuticle leads to high levels of water loss from expanding leaves (Sargent 1976; Shackel *et al.* 1987; Hamerlynck & Knapp 1996). Only once leaves fully expand can the cuticle fully develop (Hamerlynck & Knapp 1996; Hauke & Schreiber 1998). From work done in *Arabidopsis* the primary path for water loss from expanding leaves was suggested to be open stomata that were insensitive to abscisic acid (ABA) when they first developed and only became sensitive to ABA once they were exposed to dry air after which they would function normally (Pantin *et al.* 2013). This is somewhat incompatible with the concept that plants generally aim to minimize excessive water loss without any carbon gain (Wong, Cowan & Farquhar 1979), with photosynthesis being extremely low in expanding leaves. A better understanding of the timing of the development of the cuticle and stomata is critical for understanding leaf water relations in expanding leaves, and work in this area is especially needed in long lived trees to confirm the theory of stomatal functional development and cuticle formation in developing leaves proposed by work in *Arabidopsis*. Understanding water loss from developing leaves of will be the aim of Chapter 2 of this thesis.

## **1.2 Stomatal responses to drought in deciduous anisohydric species**

In deciduous trees once leaves have fully expanded and gained competently functioning stomata and fully formed cuticle a sudden drought can spell disaster for maximizing carbon gain and depending on the severity can lead to leaf, canopy, or even whole plant death (Cardoso, Batz & McAdam 2020a; Brodribb *et al.* 2021; Frei *et al.* 2022). Death during drought is caused by increasing tension in xylem conduits and if the tension becomes too negative the water column

can be invaded by air which forms an unrecoverable embolism (Skelton, Brodribb, McAdam & Mitchell 2017; Cardoso, Brodribb, Kane, DaMatta & McAdam 2020b; Brodribb *et al.* 2021). The water potential that causes embolism to form varies widely across species (Avila *et al.* 2023). Before lethal embolism formation drought closes stomata to maintain water potential higher than that which could induce embolism, this comes at the considerable cost of reduced photosynthesis (Brodribb *et al.* 2020). Trees are often classed as isohydric or anisohydric depending on how sensitive stomata are in responding to water potential decline during drought (Tardieu & Simonneau 1998; Gallé *et al.* 2013; Sade & Moshelion 2014). Isohydric species are able regulate their water loss during droughts by closing stomata with ABA, maintaining relatively constant water potentials during short term dry periods. Anisohydric species often have much larger fluctuations in water potential and are characterized by stomata that are less responsive to changes in water potential (Jones 1998; Attia, Domec, Oren, Way & Moshelion 2015). These two strategies are thought to exist along a continuum with some species exhibiting both behaviors depending on the annual precipitation of the native range of the population (Klein 2014; Leuschner 2020; Chen *et al.* 2021). Some have questioned the idea that ABA is involved in closing the stomata of anisohydric species due to the apparent insensitivity of stomatal conductance to changes in water potential in these species (Leuschner, Schipka & Backes 2022). With droughts increasing in frequency and intensity in temperate regions it is critical to understand the mechanistic regulation of stomata in deciduous species in response to drought, and to understand if anisohydric species are actively closing stomata with ABA or closing stomata passively (Tardieu & Simonneau 1998; Brodribb & McAdam 2011). Investigating this will be the aim of Chapter 3 of this thesis.

### **1.3 The role of ABA in leaf senescence**

Leaf senescence marks the end of deciduous growing seasons, during this time of the life of a leaf, nutrients from chlorophyll and other compounds are withdrawn from leaves revealing the yellow carotenoids and red anthocyanin pigments normally masked by the dark green photosynthetic pigments (Lim *et al.* 2007). Leaf senescence in deciduous trees is primarily driven by changes in daylight and temperatures (Lang, Chen, Qian, Liu & Piao 2019). While the abiotic drives of autumn leaf senescence are broadly understood the physiology of leaf senescence is still under investigation (Gan & Amasino 1997; Richardson *et al.* 2012; Liu *et al.* 2020). Most of the work done on leaf senescence focuses on annual herbs and crops like *Arabidopsis* and rice, with

much of the work being performed *ex situ* on detached leaves or leaf discs (Gepstein & Thimann 1980; Gao *et al.* 2016; Zakari, Asad, Han, Zhao & Cheng 2020). The process of senescence is very important in agriculture, with crops like soybean requiring complete senescence before harvest with some even using and developing chemicals to hasten the senescence process (Setter, Brun & Brenner 1980; Harbach *et al.* 2016). Ethylene is considered the primary hormonal driver of leaf senescence, but ABA is thought to be a secondary driver of this process (Noodén & Leopold 1988; Iqbal *et al.* 2017). ABA has been found to accelerate leaf senescence in leaf discs soaked in solutions, albeit with levels of ABA which are far higher than have been observed *in situ* (Gao *et al.* 2016). Some have observed in whole plants that ABA levels increase during leaf senescence, while others have found that foliar ABA levels decline during the senescence process (Gepstein & Thimann 1980; He, Osaki, Takebe, Shinano & Wasaki 2005; Uzelac *et al.* 2016). It has even been suggested that ABA plays an indirect role in senescence via closing stomata with reductions in photosynthesis directly driving senescence (Thimann & Satler 1979b a). In Chapter 4 of this thesis I seek to investigate the role of ABA in driving senescence timing in deciduous tree species.

#### **1.4 How leaves survive freezing**

Many deciduous plants in higher latitudes may have evolved a leaf life span that avoids exposure of photosynthetic tissues to frosts (Zanne *et al.* 2014). The leaves of annual and perennial herbs have evolved to emerge in the spring as early as possible yet avoid frosts, and then flower, set seed, and die or go dormant underground before autumn frosts begin (Clements & Ludlow 1977; Sakai & Larcher 1987; Bassow & Bazzaz 1998; Lubbe & Henry 2019). One of the primary theories of why plants evolved a deciduous habit was similarly to avoid freezing that can kill leaves and cause embolism in stems (Zanne *et al.* 2014; Edwards *et al.* 2017). Unlike many evergreen boreal species which can survive temperatures down to and possibly below -40°C, deciduous tree species can be severely damaged by temperatures of just -5°C (Burke, Gusta, Quamme, Weiser & Li 1976; Cavender-Bares *et al.* 2005; Strimbeck, Kjellsen, Schaberg & Murakami 2007). There are also many species that have an intermediate leaf habit, the leaves of species that are brevid deciduous can survive lower temperatures than typical deciduous species (Francescantonio *et al.* 2020). These species can have leaves that are lost or killed late in winter but only remain leafless for a few weeks up to two months in winter. These species provide an excellent system in

which to investigate the key traits responsible for triggering leaf death during freezing in deciduous species.

The process by which a plant is damaged by freezing can vary depending on species, minimum leaf temperature, and the rate of freezing (Sakai & Larcher 1987). Leaves that are not frost tolerant when exposed to sub 0°C temperatures will experience freezing of the cytoplasm with the resulting ice crystals puncturing the cell membrane resulting in cell death (Sakai & Larcher 1987). Plants have two primary ways of being frost tolerance (Burke *et al.* 1976; Sakai & Larcher 1987). The first is frost avoidance where cells are osmotically loaded which depresses the freezing point of the cytoplasm so that the water in the plant tissue will stay liquid when air temperatures fall below freezing. This is considered a more transient survival strategy that would allow for a plant to survive incidental, anomalous freezing events, because in these species if the depressed freezing point is passed it can result in extremely rapid damage (Thomas & Barber 1974; Sakai & Larcher 1987; Rada, Goldstein, Azocar & Torres 1987; Squeo, Rada, Azocar & Goldstein 1991; Larcher, Meindl, Ralser & Ishikawa 1991; Ball *et al.* 2002). The other frost tolerance pathway is freezing tolerance where ice will form in leaves, usually in intercellular spaces, while the cytoplasm remains liquid allowing for full recovery of cell function on warming (Sucoff, 1969; Sakai and Larcher, 1987; Sperry and Sullivan, 1992; Davis *et al.*, 1999; Cochard *et al.*, 2001; Feild and Brodribb, 2001). The primary risk for this strategy is cell dehydration, as the temperature falls and the apoplastic water freezes more symplastic water is pulled from the cell and frozen in the apoplast which can lead to cellular dysfunction and death (Stergios & Howell 1973; Sakai & Larcher 1987; Squeo *et al.* 1991; Ashworth 1993; Taschler & Neuner 2004). With climate change leading to increasing spring frost risk to deciduous trees, through an increase in the unpredictability in weather patterns, during the most sensitive times for leaf survival, it is critical to have better tools to understand how leaves freeze and what allows the leaves of some species to survive a frost event that would kill other species. Chapter 5 of this thesis addresses these key questions about freezing tolerance and leaf survival.

## **1.5 Climate change changing phenology**

Climate change is having major effect on phenology in most ecosystems across the globe (Hänninen 1995; Menzel & Fabian 1999; Cleland, Chuine, Menzel, Mooney & Schwartz 2007; Vitasse *et al.* 2011). One of the biomes that is seeing the greatest changes to phenology is

temperate deciduous forests, because much of the phenology in these forests is controlled by temperature and day length, with warmer temperatures assumed to lead to earlier leaf out and later senescence extending growing seasons and increasing the carbon sinks of these forests (Hänninen 1995; Menzel & Fabian 1999; Vitasse *et al.* 2011). Extended growing seasons have been observed in satellite imaging, free atmospheric CO<sub>2</sub> experiments (FACE), and ground observations in deciduous forests (Norby, Hartz-Rubin & Verbrugge 2003; Godbold *et al.* 2014; Norby 2021). This small silver lining to global warming has recently been challenged by high profile publications (Zani, Crowther, Mo, Renner & Zohner 2020, 2021; Zohner *et al.* 2023). In these studies remote sensing observations have found that deciduous tree leaf lifespan may be limited by an early saturation of whole plant carbon storage (Zani *et al.* 2020, 2021; Zohner *et al.* 2023). The idea that the saturation of carbon storage in plants will lead to senescence is one that originated in studies on herbaceous plants like *Arabidopsis*, rice, and soybean (Mondal, Brun & Brenner 1978; Biswas & Mondal 1986; Noodén & Leopold 1988; Thomas 2013; Yu, Lo & Ho 2015). The recent work speculates that deciduous trees are already at or near a maximum leaf lifespan required for saturating the whole plant carbon sink each year and that early leaf out and higher early season photosynthesis will lead to early leaf senescence meaning that total leaf lifespan will remain largely unchanged or will shorten into the future, and that deciduous forests are already capturing as much carbon as they are capable of sequestering (Zani *et al.* 2020, 2021; Zohner *et al.* 2023). These results remain controversial (Norby 2021; Lu & Keenan 2022). It is vital to better understand how deciduous forest phenology will change in both the spring and autumn, as the climate warms so that we have the most accurate possible forecasts for future forest phenology and CO<sub>2</sub> sequestration in these ecosystems. Chapter 6 of this thesis presents results from an intercontinental study that addresses these questions.

## **1.6 Structure of this thesis**

In this thesis I attempt to reexamine a variety of theories and assumptions that have been made about deciduous tree leaf life span from work done primarily in herbs and annual crops plants. My work spans the breadth of the winter deciduous leaf ontogeny from bud burst to primary productivity through abiotic stress, to senescence, abscission, and leaf death. The work presented here is critical to better understanding how temperate deciduous forests will adjust and change as

the Earth's climate continues to be impacted by human induced climate change. In addressing these aims the following research questions were investigated:

- Are expanding leaves actively transpiring through stomata during development? (Chapter 2)
- What are the proportions of water lost from cuticular and stomatal pathways during development of red oak leaves? (Chapter 2)
- What role is ABA playing controlling water loss in developing leaves? (Chapter 2)
- Are anisohydric tree species actively closing stomata with ABA during drought? (Chapter 3)
- Do leaf ABA levels increase during senescence in winter deciduous trees? (Chapter 4)
- Does an unnatural increase in leaf ABA levels during autumn increase senescence rates? (Chapter 4)
- How do brevideciduous leaves survive repeated freezing events? (Chapter 5)
- What temperature threshold will kill brevideciduous leaves? (Chapter 5)
- Will warmer winters lead to longer growing seasons in deciduous forests? (Chapter 6)
- What is the breadth of phenological diversity in deciduous forests? (Chapter 6)

The five experimental chapters in this thesis are composed of self-contained units, in the style of scientific journal articles. Chapters 2-5 have been published or are currently under-review. Chapter 6 is a critical commentary of a paper recently published in *Science* in 2023 this chapter contains original data as well as a brief synthesis of literature on the subject. Chapter 7 is a concluding discussion of the findings of the experimental chapters and their implications to the relevant fields of research.

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## CHAPTER 2. A PERMEABLE CUTICLE, NOT OPEN STOMATA, IS THE PRIMARY SOURCE OF WATER LOSS FROM EXPANDING LEAVES

The text and results of this chapter are taken directly from the following publication:

Kane, C. N., Jordan, G. J., Jansen, S., & McAdam, S. A. (2020). A permeable cuticle, not open stomata, is the primary source of water loss from expanding leaves. *Frontiers in Plant Science*, 11, 774.

### 2.1 Abstract

High rates of water loss in young, expanding leaves have previously been attributed to open stomata that only develop a capacity to close once exposed to low humidity and high abscisic acid (ABA) levels. To test this model, we quantified water loss through stomata and cuticle in expanding leaves of *Quercus rubra*. Stomatal anatomy and density were observed using scanning electron microscopy. Leaves of *Q. rubra* less than 5 days after emergence have no stomata, therefore water loss from these leaves must be through the cuticle. Once stomata develop, they are initially covered in a cuticle and have no outer cuticular ledge, implying that the majority of water lost from leaves in this phase of expansion is through the cuticle. Foliar ABA levels are high when leaves first expand and decline exponentially as leaves expand. Once leaves have expanded to maximum size, ABA levels are at a minimum, an outer cuticular ledge has formed on most stomata, cuticular conductance has declined, and most water loss is through the stomata. Similar sequences of events leading to stomatal regulation of water loss in expanding leaves may be general across angiosperms.

### 2.2 Introduction

Expanding leaves are highly sensitive to abiotic stresses including drought stress (Hsiao & Xu 2000; Pantin, Simonneau & Muller 2012). Yet, somewhat paradoxically, there are reports of extremely high rates of evaporation from young, expanding leaves (Pantin *et al.* 2013). High rates of water loss in young leaves has been attributed to open stomata that are unable to close because they lack sensitivity to abscisic acid (ABA) (Pantin *et al.* 2013). A major assumption in this model is that the physical characteristics of expanding leaves are similar to those of fully developed leaves. However, several factors challenge this assumption. Cell turgor dynamics are different

between expanding and fully developed leaves, with expanding leaves maintaining high cell turgor essential for both cell expansion and the supply of nutrients to developing tissues (Shackel, Matthews & Morrison 1987; Hsiao & Xu 2000; Liu, Jensen & Andersen 2003; Siebrecht, Herdel, Schurr & Tischner 2003; Sansberro, Mroginski & Bottini 2004). Cell walls in expanding leaves must be highly flexible to allow for cell expansion (Schultz & Matthews 1993), but normal stomatal function requires rigid cell walls (Buckley, Mott & Farquhar 2003). In addition, the cuticle, a waxy layer that forms on the outer wall of the epidermal cells of all terrestrial plants (Raven 1984; Gülz 1994; Schreiber & Riederer 1996), has been dismissed as a major source of water loss in expanding leaves (Pantin *et al.* 2013). However, cuticular conductance can be very high in very young leaves and decreases during leaf expansion (Hamerlynck & Knapp 1996; Hauke & Schreiber 1998). This may reflect changes in the cuticle during leaf expansion: during the initial phase of rapid epidermal cell expansion the cuticle remains thin, elastic and often disjointed with epidermal cell shaped pieces of cuticle sitting on top of epidermal cells (Sargent 1976). Once leaf expansion ceases, the cuticle thickens, completely covering the leaf surface, while becoming firm and rigid (Sargent 1976; Onoda, Richards & Westoby 2012).

The evolution of the cuticle is believed to have allowed the aquatic algal ancestors of land plants to colonize terrestrial environments (Raven 1984; Edwards, Abbott & Raven 1996; Kenrick & Crane 1997). Despite being present on all terrestrial plants, the cuticle can vary markedly in thickness, composition, and conductance at the interspecific level, and across various developmental stages and organs within an individual plant (Jeffree 1996; Goodwin & Jenks 2005; Buschhaus, Herz & Jetter 2007; Fernández, Guzmán-Delgado, Graça, Santos & Gil 2016). Being predominantly hydrophobic wax, fully developed cuticles provide near-water tight seal on the outside of cell walls, protecting internal tissues from desiccation, blocking UV light, and acting as barrier against pathogens and physical abrasion (Edwards *et al.* 1996; Krauss, Markstädter & Riederer 1997; Łaźniewska, Macioszek & Kononowicz 2012)

Recent work suggests that cuticular organic compounds are formed within epidermal cells and transported to the outside of the cell wall via transport proteins, after which the cuticle self-assembles by evaporation (Lee & Priestley 1924; Neinhuis, Koch & Barthlott 2001; Schreiber 2005; Yeats & Rose 2013). While cuticles are deposited by evaporation, they also create an almost gas-tight seal around the cells (Lendzian 1982; Lendzian & Kerstiens 1991). The low permeability to gases severely limits CO<sub>2</sub> diffusion, which provided a strong selective pressure for the evolution

of stomata, the epidermal valves that provide internal photosynthetic cells with access to atmospheric CO<sub>2</sub> (Lendzian 1982; Lendzian & Kerstiens 1991; Brodribb, Sussmilch & McAdam 2020).

A waterproof cuticle punctuated with stomatal valves to facilitate gas exchange is essential for homoiohydric and plant growth in the desiccating environments that almost all vascular plants occupy (Lendzian 1982; Raven 1984; Brodribb *et al.* 2020). In a hydrated plant, stomata account for more than 99% of total water loss from a leaf, but once stomata close during a drought, it is believed that a considerable proportion of water lost from the plant evaporates via the cuticle (Körner 1994; Duursma *et al.* 2019). After drought-induced closure of stomata, between 50 and 94% of the water lost from leaves is reported to be lost through the cuticle or incompletely closed stomata (Šantrůček, Šimánková, Karbulková, Šimková & Schreiber 2004; Brodribb, McAdam, Jordan & Martins 2014). Much like the variation in maximum stomatal conductance (Körner 1994), the degree of variation in cuticular conductance between species can be considerable and may be critical for determining the ecological limits of species (Schreiber & Riederer 1996; Mayr 2007). Highly permeable cuticles are found in moss and fern gametophytes, while very low cuticular conductance is found in species that are adapted to dry environments, determining drought tolerance across species (Edwards *et al.* 1996; Jeffree 1996; Schreiber & Riederer 1996; Brodribb *et al.* 2014; Blackman *et al.* 2016; Lee *et al.* 2019; Carignato, Vázquez-Piqué, Tapias, Ruiz & Fernández 2020). Pollutants and time can degrade leaf cuticle impacting drought resistance (Jordan & Brodribb 2007; Burkhardt & Pariyar 2014). In particular, the removal of outer cuticular waxes can severely decrease drought tolerance in semiarid woody species, leading to a reduction in photosynthesis, gas exchange, and plant pigment levels (Medeiros *et al.* 2017; Pereira, Figueiredo-Lima, Oliveira & Santos 2019).

Although there has long been a focus on cuticular conductance in determining drought-tolerance thresholds, almost no focus has been placed on the role of cuticular conductance in determining leaf gas exchange as leaves expand. Complete leaf expansion in *Hedera helix* occurs around the same time cuticular conductance reaches a minimum (Hauke & Schreiber 1998). Cuticles also appear to cease developing in chemical composition once leaves cease expanding (Hauke & Schreiber 1998). Furthermore, very young stomata are covered in a cuticle (Davis & Gunning 1992; Nadeau & Sack 2002; Hunt *et al.* 2017). Breaking of this cuticle covering later in leaf development to form the outer cuticular ledge may be responsible for reported increased in

leaf gas exchange as leaves expand (Constable & Rawson 1980). In support of this is that rates of gas exchange in mutant plants of *Arabidopsis* stomata occluded by a cuticle covering are half that of wild-type plants without occluded stomata (Hunt *et al.* 2017).

Here, we utilize the hypostomatic species *Quercus rubra* to separate cuticular and stomatal water loss from total leaf transpiration in expanding leaves. *Q. rubra* has large, fast-growing leaves making it ideal for these experiments. We reexamine the ontogeny of the formation of the outer cuticular ledge in expanding *Arabidopsis* leaves, which is essential for the initiation of stomatal conductance. We also collected foliage ABA levels in expanding leaves to examine what, if any, role ABA may play in ‘priming’ stomatal function.

## 2.3 Methods

### 2.3.1 Plant material

Six, three-year-old bare-rooted *Quercus rubra* plants were planted in 10 l pots containing a 1:1:1 mix of Indiana Miami topsoil, ground pine bark, and sand. Plants were grown in the glasshouses of Purdue University, IN, USA under a 16 h photoperiod, supplemented and extended with LED lights (Illumitex Power Harvest I4, TX, USA) that provided a photon flux density on an F3 spectrum (22.4% blue; 13.4% green; 63.9% red; 0.4% far-red) of  $150 \mu\text{mol m}^{-2} \text{s}^{-1}$  at pot level. The highest PPFD (natural and supplemental light) measured  $1800 \mu\text{mol m}^{-2} \text{s}^{-1}$  at solar noon on a cloudless day. Plants were watered daily and received liquid nutrients once per month. Conditions in the glasshouse were set at a night/day temperature of 22/28°C. After initial bud burst all developing leaves were tagged with the date of leaf emergence. Six plants of *Arabidopsis thaliana* Col-0 were grown under a 10 h photoperiod, supplied by LED lights (SUNCO Lighting, CA, USA), providing a photon flux density of  $60 \mu\text{mol m}^{-2} \text{s}^{-1}$  at pot level. Seeds were sown directly on germination mix (Sun Gro Horticulture, MA, USA). Plants were watered from the base and given liquid nutrients once per month. Plants were imaged daily to determine leaf age. The area of eight leaves was measured daily from initial emergence until 23 days after emergence.

### 2.3.2 Determining cuticular and stomatal conductance by leaf gas exchange

Leaf gas exchange was measured using an infrared gas analyzer (LI-6800, Licor Biosciences, NE, USA). Conditions in the leaf cuvette were maintained as close to ambient

glasshouse conditions as possible, and light conditions were set at  $1500 \mu\text{mol m}^{-2} \text{s}^{-1}$ . Measurements were taken between 09:00 till 11:00 on clear, cloudless days. Initial stomatal conductance ( $g_s$ ) was measured on expanding, or fully expanded, leaves by enclosing the leaf in the chamber and measuring instantaneous leaf gas exchange parameters. After this initial measurement the abaxial surface of the leaf was covered in petroleum jelly and plastic wrap and instantaneous leaf gas exchange was again measured in the same region of the leaf, or the whole leaf. By covering the abaxial leaf surface we are only measuring gas exchange through the adaxial surface which has no stomata or hydathodes, like most *Quercus* species (Bolhàr-Nordenkamp & Draxler 1993). All rates of leaf gas exchange were normalized by leaf area in the cuvette. Whole leaf area was also measured for each leaf analyzed by imaging leaves (12 megapixel, iPhone 7, Apple Inc, CA, USA) and measuring area using ImageJ (National 303 Institutes of Health, Bethesda, MD, USA). To avoid variation due to potential developmental variation across the leaf surface the center of each leaf was placed in the cuvette. In younger leaves we were able to measure the whole leaf. All measured leaves were preserved in methanol and stored at  $-20^\circ\text{C}$  for anatomical assessment. Cuticular and stomatal conductance, and percent of total leaf conductance that occurred through the stomata were calculated according to Jordan & Brodribb (2007).

### 2.3.3 Quantifying foliage abscisic acid levels

Leaves were harvested at 11:00 and immediately wrapped in damp paper towel and bagged. A sample of tissue was taken from each leaf, weighed ( $\pm 0.0001\text{g}$ , OHAUS Corporation, NJ, USA) and then covered in  $-20^\circ\text{C}$  80% methanol in water ( $v v^{-1}$ ) containing  $250 \text{ mg l}^{-1}$  butylated hydroxytoluene, chopped to fine pieces and stored at  $-20^\circ\text{C}$  overnight. Extraction in methanol ensures that both free and fettered ABA in the chloroplasts was extracted from the sample (Georgopoulou & Milborrow 2012). The samples were homogenized and  $15 \mu\text{l}$  of deuterium labelled [ $^2\text{H}_6$ ]ABA (OIChemim ltd, Czech Republic) was added as an internal standard. Absciscic acid (ABA) was extracted overnight at  $4^\circ\text{C}$ . An aliquot of supernatant was dried in a vacuum sample concentrator (Labconco, MO, USA) and ABA was resuspended in  $200 \mu\text{l}$  of 2% acetic acid in water ( $v v^{-1}$ ), centrifuged at 14,800 RPM for 4 minutes and  $100 \mu\text{l}$  taken for analysis. The level of ABA and internal standard in each sample was quantified using an Agilent 6460 series triple quadrupole LC/MS (Agilent, CA, USA) according to McAdam (2015). After quantification, the

plant material from which the supernatant was taken was dried down at 70°C, and leaf dry weight was estimated by subtracting the initial mass of the empty tube.

#### 2.3.4 Anatomy

Stomatal anatomy was analyzed in hole punches (diameter 0.5cm) from the center of *Q. rubra* leaves ranging from 1 to 30 days of age (including all of the leaves measured for leaf exchange) that had been stored in methanol at -20°C. Anatomical samples were collected from either the whole leaf, in young leaves or from center of the leaves when they were large enough. In *Q. rubra* leaves expand evenly and then acropetally after reaching approximately 70% of maximum size (Tomlinson, Dickson & Isebrands 1991), our sampling protocol ensured that we avoided these regions of differential or continual expansion in larger leaves. Samples were prepared for SEM by critical point drying (E3000 Critical Point Dryer, Quorum Technologies, East Sussex, UK). Dried samples were placed on stubs and sputter coated for 60s at 8 mA using a gold target (Balzers Union FL-9496 sputter device, Balzers, Liechtenstein). Images of stomata from the abaxial surface were taken on a Phenom XL desktop SEM (Nano Science Instruments, AZ, USA) at 1,000x magnification to determine stomatal density, and the percent of stomata in which the outer cuticular ledge had formed. For stomatal density measurements, a stoma was counted if both guard cells were discernible. A stoma with an outer cuticular ledge was defined as having any form of rip, tear, or hole in the cuticular covering over the stomatal pore. Cross sections of *Q. rubra* leaves were made using a freezing microtome (Microm HM 430, Thermo Scientific, MA, USA). The cuticle on leaf sections was stained using Sudan IV (0.5g powdered Sudan IV in 100ml 75% Ethanol, 25% DI water) for 8 hours at 25°C. Images were taken using a 40x oil emersion objective on a light microscope (AxioImagerA2, Zeiss, Germany). Observations were made from four different sections from three different leaves six and 21 days after emerging.

*Arabidopsis* leaves used for stomatal anatomy were harvested on a single day and stored in methanol at -20°C. Leaf segments were prepared to observe the abaxial leaf surface and attached to a SEM stub with 1:1 OCT cryo-gel and water. Leaf pieces were frozen in a liquid nitrogen slurry and moved into a Gatan Alto 2500 (Gatan 316 Inc., Pleasanton, CA, USA) cryo-preparation chamber of an SEM (FEI Nova Nano 317 200, Hillsboro, OR, USA). The samples were placed under vacuum and held at -170°C. Samples were then allowed to sublime at -90°C,

while viewing to remove frost. Leaves were sputter coated for 120 s at 8 mA using a platinum target and then imaged at -140°C.

### 2.3.5 Leaf water potential

Midday leaf water potential was measured in young expanding leaves (six days after leaf emergence), as well as fully expanded leaves (32 days after leaf emergence) using a Scholander pressure chamber (PMS Instrument Company, OR, USA). Leaves were excised and wrapped in damp paper towel and immediately placed into humid plastic bag. Leaves were allowed to equilibrate in dark, in the humid bag for 5 minutes before measurements were taken.

## 2.4 Results

In the newest expanding leaves of *Q. rubra* (less than 5 days old; i.e. at ~ 15% of fully expanded area), whole leaf conductance was found to be relatively high, at  $0.023 \text{ mol m}^{-2} \text{ s}^{-1}$ . By 10 days after leaf emergence (i.e. at 60% of fully expanded area), leaf conductance had doubled to  $0.047 \text{ mol m}^{-2} \text{ s}^{-1}$  (Figure 2-1; Figure 2-2). While leaf conductance was measurable in leaves that were less than 5 days old, less than 5% of total leaf conductance was found to be lost through the stomata (Figure 2-1). After 5 days of leaf expansion, the percentage of water lost from a leaf through stomata began to increase rapidly (Figure 2-1). 10 days after leaf emergence, the stomata were found to be responsible for approximately 50% of water loss from the leaf (Figure 2-1). By 15 days after leaf emergence, the percentage of water lost through the stomata accounted for more than 80% of total leaf conductance, which had increased to more than  $0.075 \text{ mol m}^{-2} \text{ s}^{-1}$  (Figure 2-1). By this age leaves were fully expanded. In general, leaves had ceased to expand by day 13 (Figure 2-2). Leaves 6 days after emerging did not appear to have a very thick or well-developed cuticle when compared to leaves 21 days after emerging, which displayed a much thicker and well-developed cuticle (Figure 2-1).

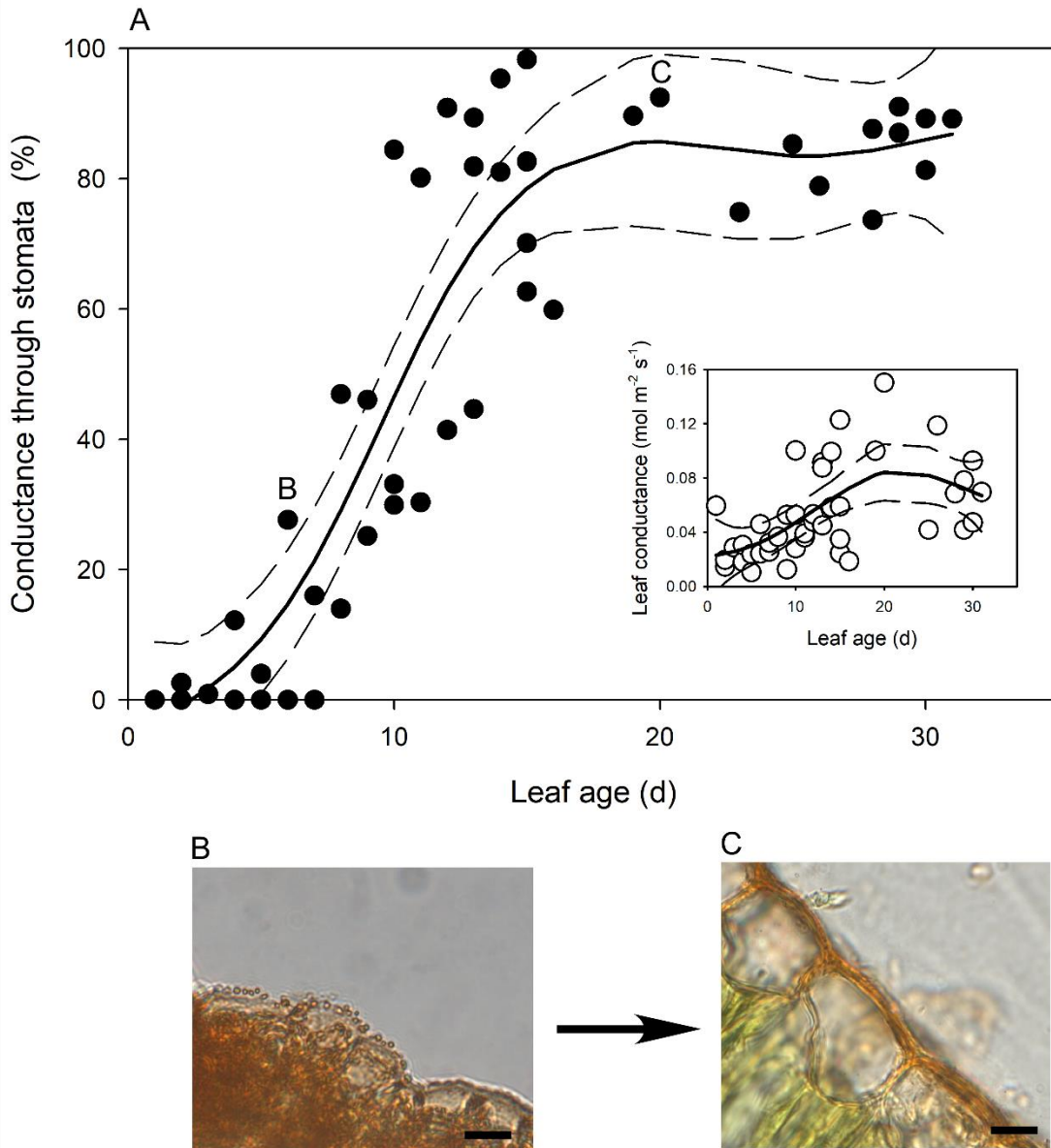


Figure 2-1. (A) The percentage of transpired water lost through stomata as *Quercus rubra* leaves expand. The insert depicts the absolute rates of leaf conductance measured in the same leaves. Generalized additive model curves and 95% confidence intervals are represented by solid and dashed black lines, respectively. Each point represents a single leaf. Letters on the chart depict the leaf from which representative images B and C were taken. (B) Cross sections through the epidermis of a *Q. rubra* leaf 6 days after emerging, and (C) 21 days after emerging, with cuticles stained with Sudan IV (scale bars = 10 $\mu$ m).



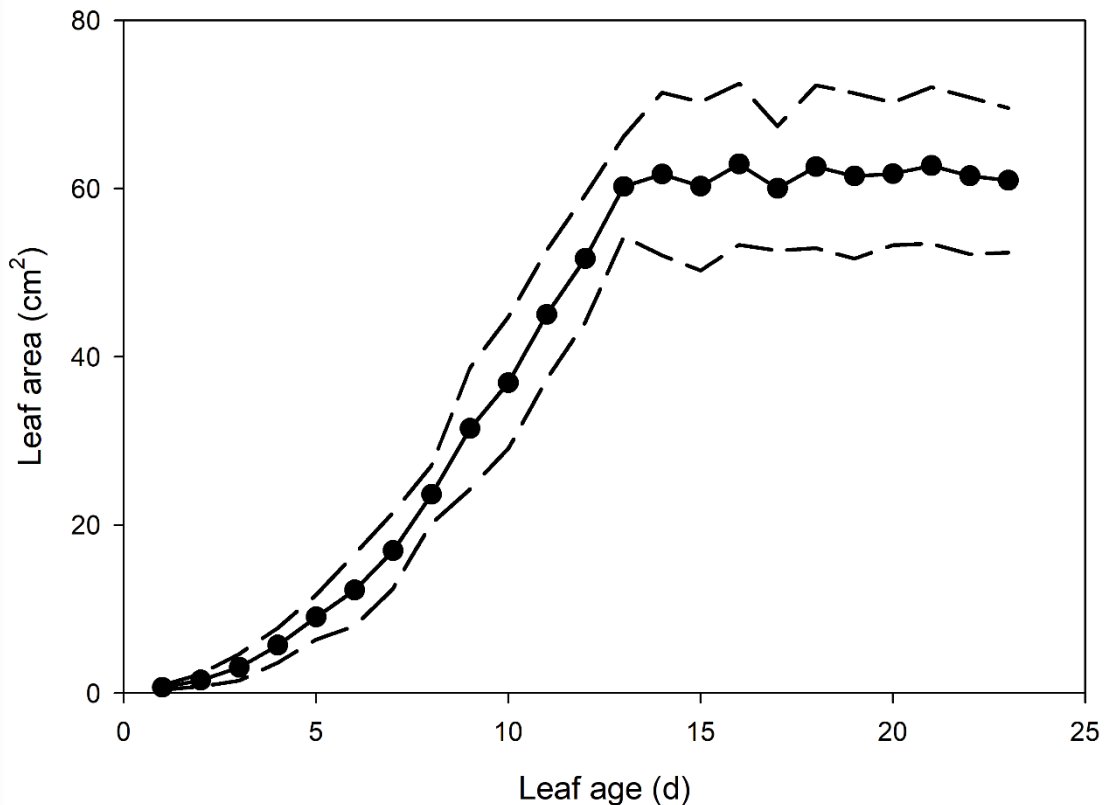


Figure 2-2. Mean leaf area of *Quercus rubra* leaves from emergence (day 0) to 23 days after leaf emergence ( $n = 8$  leaves,  $\pm$  SD). Dashed lines depict standard deviation.

Foliar ABA levels in developing *Q. rubra* leaves were approximately  $21.5 \mu\text{g g}^{-1}$  dry weight on the first day following leaf emergence (Figure 2-3). As leaves expanded, this high level of initial ABA in primordial leaves declined following an exponential decay curve, such that by seven days after leaf emergence, ABA levels in terms of dry weight were half the initial level in the newest emerged leaves (Figure 2-3). ABA levels continued to decline until around 30 days after initial leaf emergence, by which time they had approached a steady-state level of around  $0.55 \mu\text{g g}^{-1}$  dry weight (Figure 2-3).

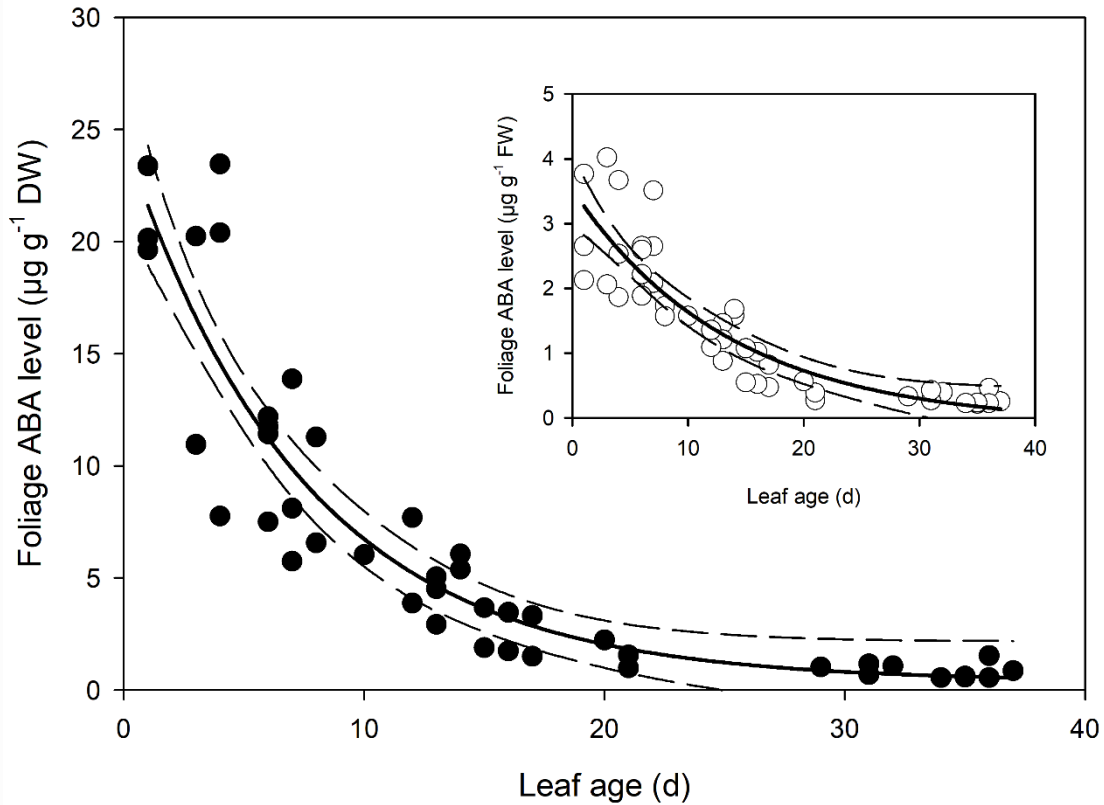


Figure 2-3. Foliage abscisic acid (ABA) level in expanding *Quercus rubra* leaves. ABA levels are expressed in terms of dry weight. A single exponential decay 3 parameter model (ABA level DW =  $0.3822 + 24.2829 \times e^{-0.1340 \times \text{Leaf age}}$ ) (solid line) with 95% confidence interval (dashed lines) is depicted ( $P = <0.0001$ ,  $R^2 = 0.8493$ ). The insert shows ABA levels in terms of fresh weight (FW). A single exponential decay 3 parameter models (ABA level FW =  $-0.0982 + 3.6244 \times e^{-0.0737 \times \text{Leaf age}}$ ) (solid line) and 95% confidence interval (dashed line) are depicted ( $P = <0.0001$ ,  $R^2 = 0.7912$ ).

The youngest *Q. rubra* leaves had very few stomata, with approximately  $27 \pm 2$  stomata  $\text{mm}^{-2}$  by the second day following emergence (Figure 2-4). Stomatal densities remained low in expanding leaves until five days after leaf emergence, when densities rapidly increased by 20-fold, to approximately 575 stomata  $\text{mm}^{-2}$  (Figure 2-4). Allowing for a change in leaf area, this indicates a 200 000-fold increase in the total number of stomata over that time (Figure 2-4). The highest recorded stomatal density on an individual leaf was measured in leaves 9 days after leaf emergence, with  $1,528 \pm 33$  stomata  $\text{mm}^{-2}$  (Figure 2-4), after which stomatal density declined as leaves continued to expand. Seventeen days after leaf emergence, stomatal density reached a steady-state mean density of 790 stomata  $\text{mm}^{-2}$  ( $\pm 5$ ) (Figure 2-4).

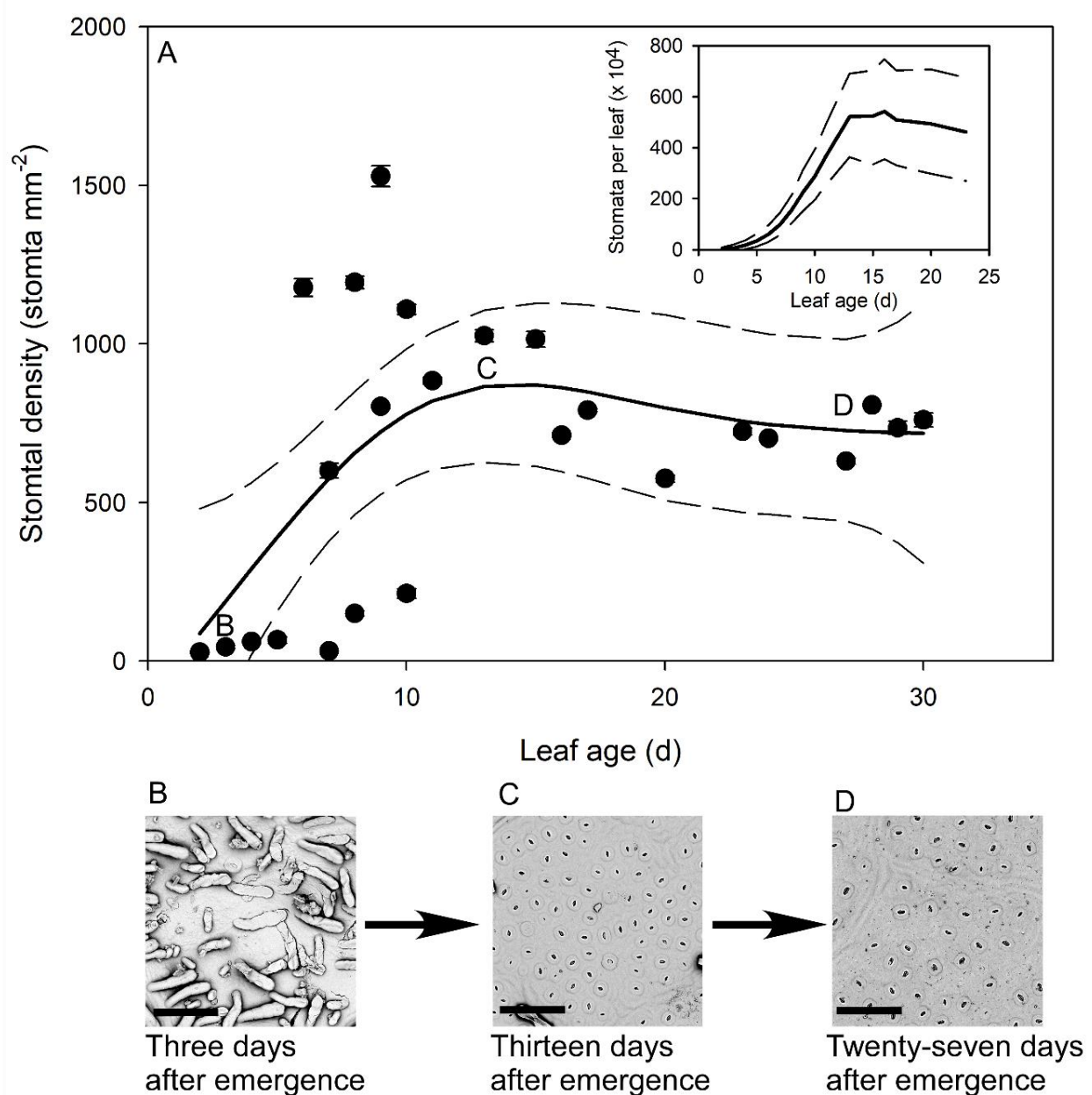


Figure 2-4. (A) Mean stomatal density ( $n = 5$  fields of view per leaf taken from the center of the leaf,  $\pm$ SE) of expanding *Quercus rubra* leaves. Each point represents a single leaf. Letters on the chart depict the leaf from which representative images B, C and D were taken. Generalized additive model curves and 95% confidence intervals are represented by solid and dashed black line respectively. The insert represents the total number of stomata per leaf of expanding *Quercus rubra* leaves (solid line) flanked by the 95% confidence interval (dashed line). (B) An image of the abaxial surface of a *Quercus rubra* leaf three days after emergence with visible trichomes (scale bar = 80 μm). (C) An image of the abaxial surface of a *Quercus rubra* leaf thirteen days after emergence with (scale bar = 80 μm). (D) An image of the abaxial surface of a *Quercus rubra* leaf twenty-seven days after emergence (scale bar = 80 μm).

In all stomatal complexes on leaves younger than seven days old a cuticle covered the pore between the guard cells (Figure 2-5). The presence of this covering meant that these stomatal complexes did not have apertures and therefore could not be functional stomata. By 13 days after leaf emergence, in 90% of stomatal complexes this cuticle layer had split to create an aperture and an outer cuticular ledge (Figure 2-5). Similar patterns in the formation of the outer cuticular ledge were observed in the expanding leaves of *Arabidopsis thaliana Col-0* plants (Figure 2-6; Figure 2-7) with most stomata in the smallest and youngest leaves covered with cuticle (Figure 2-7). 0-5% of stomata had formed an outer cuticular ledge in leaves of *A. thaliana* that were  $<0.25\text{mm}^2$  in area and had not yet emerged from the center of the rosette. Once leaves had emerged from the rosette for approximately one day (being more than  $10\text{ mm}^2$  in area), approximately 25% of the stomata had developed an outer cuticular ledge (Figure 2-7). The number of stomata forming an outer cuticular ledge per day declined once *A. thaliana* leaves reached approximately  $15\text{mm}^2$  in area.

We found that leaf water potential of young expanding leaves was the same as that of fully expanded leaves on the same plant. Leaves three days after emerging had a water potential of  $-0.866 \pm 0.113\text{ MPa}$  ( $n=3$ , SE) while leaf water potential in leaves that emerged at least 32 days prior to the measurement, and were fully expanded, was  $-0.763 \pm 0.089\text{ MPa}$  ( $n=3$ , SE).

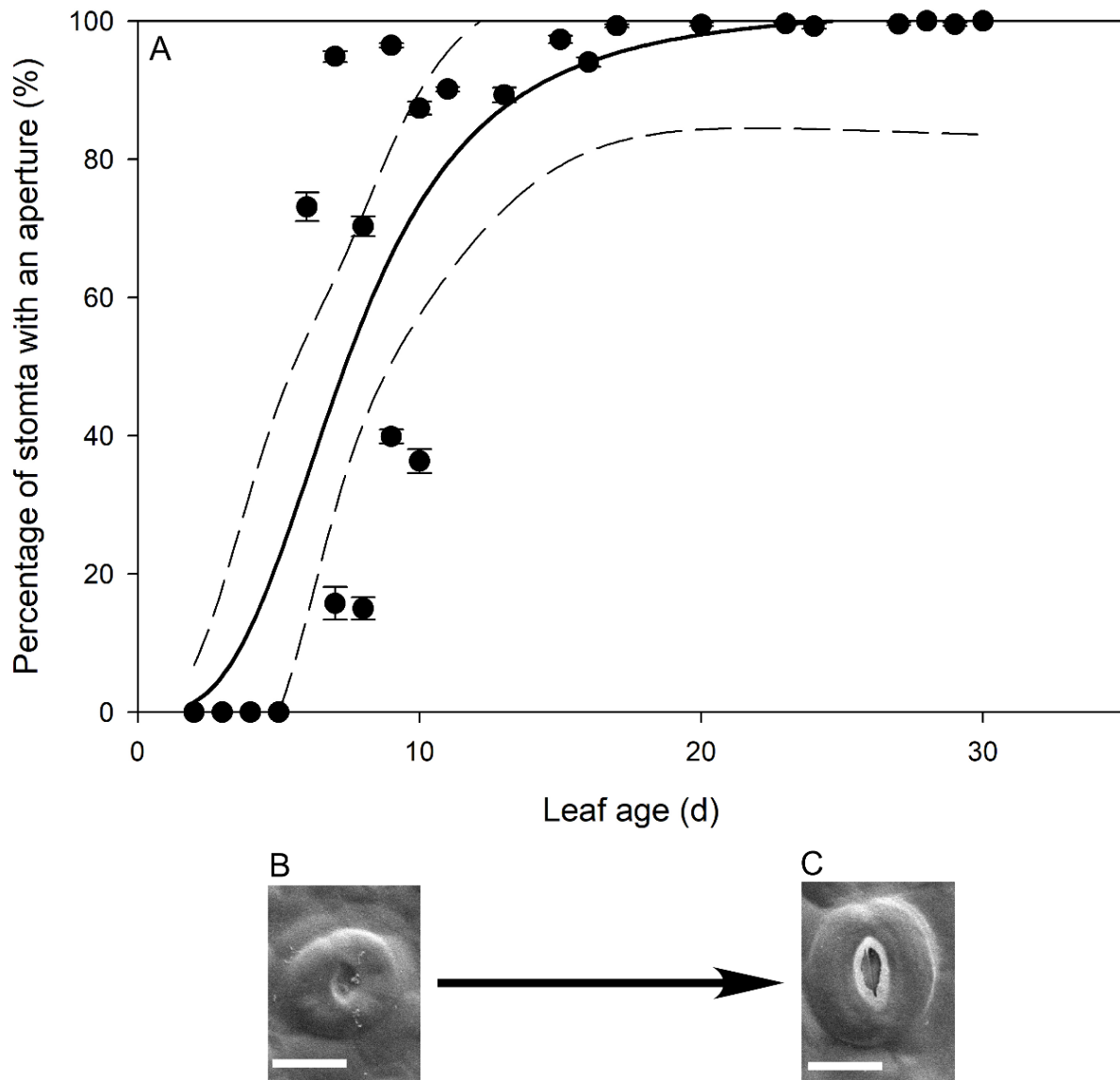


Figure 2-5. (A) Mean percentage of stomata with an aperture ( $n = 5$  fields of view per leaf taken from the center of the leaf,  $\pm$  SE) in expanding leaves of *Quercus rubra*. Each point represents a single leaf. A logistic 3 parameter sigmoidal curve (solid line) and 95% confidence interval (dashed line) is shown ( $P = <0.0001$ ,  $R^2 = 0.7178$ ). (B & C) Representative images of *Quercus rubra* stomata (B) without an aperture and (C) with an aperture captured on the same leaf 10 days after emergence (scale bar = 10  $\mu$ m).

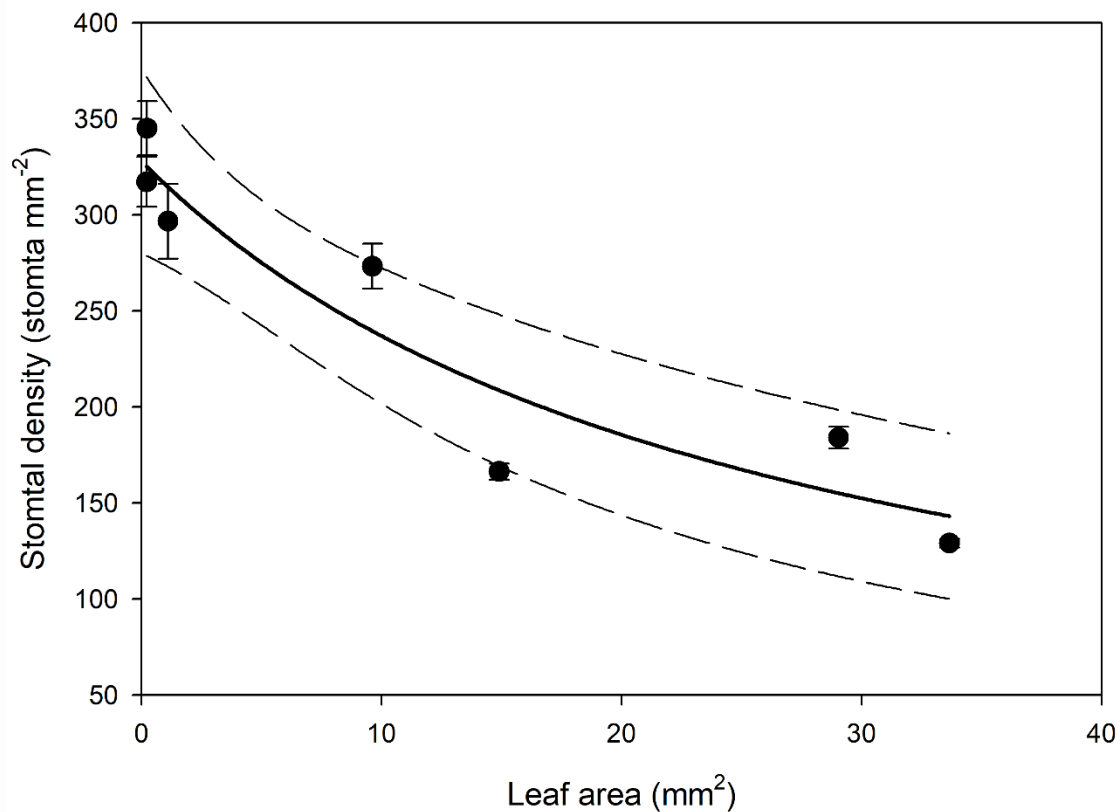


Figure 2-6. Mean stomatal density on the abaxial surface ( $n = 5$  fields of view from the same leaf taken from the center of the leaf,  $\pm$  SE) in expanding *Arabidopsis thaliana Col-0* leaves. Each point represents a single leaf. A Rational, 2 Parameter II curve (solid line) and 95% confidence interval (dashed line) is shown ( $P = <0.0015$ ,  $R^2 = 0.8870$ ).

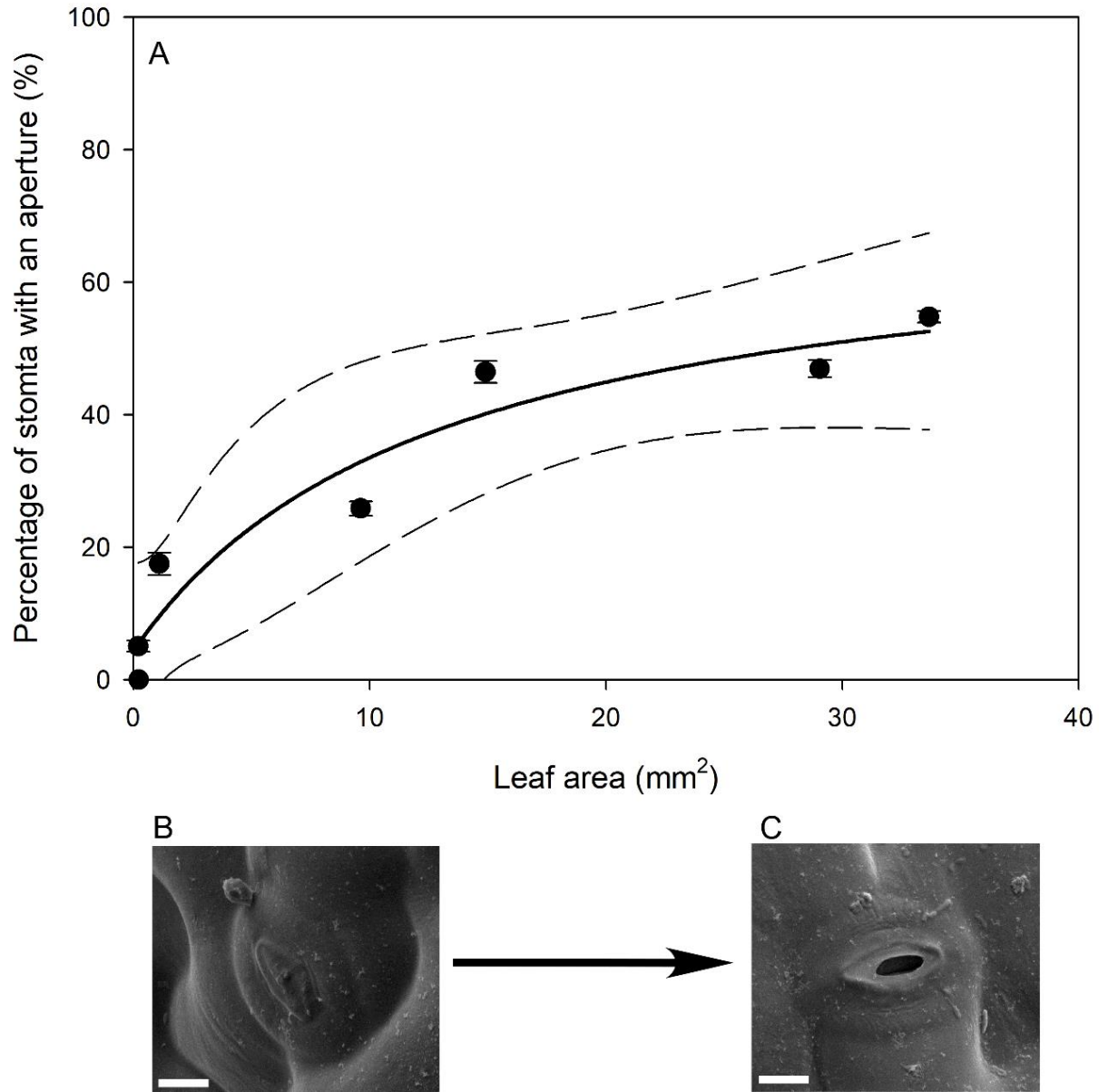


Figure 2-7. (A) Mean percentage of stomata that have formed an aperture on the abaxial surface ( $n = 5$  fields of view per leaf taken from the center of the leaf,  $\pm$  SE) in young expanding leaves of *Arabidopsis thaliana Col-0*. Each point represents a single leaf. A Rational, 3 Parameter II (solid line) and 95% confidence interval (dashed line) is shown ( $P = <0.0050$ ,  $R^2 = 0.9295$ ). (B) Image of an *A. thaliana Col-0* stoma without an aperture on a leaf that was 29.04 mm<sup>2</sup>, approximately 6 days after emergence (Scale bar = 5  $\mu$ m). (C) Image of an *A. thaliana Col-0* stoma with an aperture on with the same leaf imaged in B (Scale bar = 5  $\mu$ m).

## 2.5 Discussion

Contrary to the model of Pantin *et al.* (2013) based on observations in *Arabidopsis*, cuticular conductance accounts for the majority of water loss from expanding leaves in *Q. rubra*. In *Q. rubra* the youngest leaves have no stomata and once stomata form, they have no aperture as they are still covered in cuticle. Only once the stoma and aperture forms by tearing the covering cuticle do stomata become the primary source of leaf conductance to water vapor. We found no evidence in *Q. rubra* that ABA levels increased as leaves expand, thereby priming stomata to function as hypothesized by Pantin *et al.* (2013). In contrast, ABA levels were very high in young expanding leaves and appeared to decline thereby presumably allowing stomata to open.

The highly permeable cuticle in young, expanding leaves previously observed in *Q. macrocarpa*, *Q. muehlenbergii*, and *Hedera helix* (Hamerlynck & Knapp 1996; Hauke & Schreiber 1998) may be due to the development of the cuticle (Lee & Priestley 1924; Neinhuis *et al.* 2001). Mature cuticles are extremely dense with a very high breakage strength, suggesting that a weaker cuticle may be necessary to allow cells and leaves to expand (Onoda *et al.* 2012). The more elastic disjointed developing cuticle needed to allow cell expansion may come at the cost of a higher cuticular conductance. If this is the case, plants would have to balance the maintenance of high turgor pressure to drive cell expansion and deliver nutrients with a permeable cuticle to allow for cell expansion. Although cuticle permeance has been found to be a function of water status with high leaf water potential leading to higher levels of cuticular water loss (Boyer, Wong & Farquhar 1997; Jordan & Brodribb 2007), it is unlikely that the high levels of cuticular water loss in young leaves might simply be due to the higher water status of young expanding leaves as these leaves have the same water potentials as fully expanded leaves. This is in agreement with previous work in other *Quercus* species, in which there was no difference found in leaf water potential across leaf age as leaves expand (Ren & Sucoff 1995; Hamerlynck & Knapp 1996). In *Q. rubra* we observed much thinner cuticles in younger leaves when compared to those that were fully expanded, this anatomical change in cuticle thickness and possibly composition, is the likely cause of the higher cuticular water loss measured in young expanding leaves.

Our work suggests the formation of the outer cuticular ledge above stomata of developing leaves (and therefore formation of an aperture) could be a major determinant of the timing and relevance of stomatal function in leaf gas exchange. Here, we observed that stomatal water loss only occurs when stomata have these apertures (Figures 2-1 and 2-4). The cuticle that covers



stomata before the formation of the outer cuticular ledge likely inhibits water flux through individual stomatal pores, just as it reduces stomatal conductance in *A. thaliana* mutant plants that do not form an outer cuticular ledge (Hunt *et al.* 2017). Once that cuticle tears and the outer cuticular ledge is formed, *Q. rubra* stomata are capable of sustaining maximum water loss rates through the pore. These cuticle coverings in young stomata have been observed multiple times in *A. thaliana* (Serna & Fenoll 1997; Nadeau & Sack 2002; Hunt *et al.* 2017), in *Hydrocotyle bonariensis* (Koch & Barthlott 2009), the stomata on the flowers of *Vicia faba* (Davis & Gunning 1992), and now *Q. rubra*. Given that we observed these in both *Q. rubra* and *A. thaliana*, and stomatal development and developmental genes are highly conserved across land plants, this cuticular covering of young stomata may be a feature common to all vascular plants (Chater, Caine, Fleming & Gray 2017). Whether it extends to non-vascular plant stomata remains to be examined (Renzaglia, Villarreal, Piatkowski, Lucas & Merced 2017).

The extremely high levels of ABA found in young leaves of *Q. rubra* could have several explanations all requiring future examination. It is possible that the newest expanding leaves have high levels of ABA because ABA is required to maintain bud dormancy (Kovaleski & Londo 2019). The decreases seen here as leaves expand might be due to dilution and catabolizing as bud dormancy is broken (Kovaleski & Londo 2019). The ABA may also be playing a role in cuticle formation, as some ABA deficient tomato mutants have thinner cuticles with reduced levels of cutin that are partially restored by the application of ABA (Martin, Romero, Fich, Domozych & Rose 2017). Another possibility is that ABA may be responsible for maintaining low guard cell turgor during leaf development to stop the premature tearing of the cuticle covering above the stomatal pore. Exogenous applications of ABA have been found to keep stomata closed under the cuticle covering in *focl* mutants, which have much reduced formation of the outer cuticular ledge, indicating that stomata that have a cuticle covering are possibly capable of opening and closing (Hunt *et al.* 2017). There is the possibility that the high levels of ABA in young leaves may be sequestered in chloroplasts, this fettered ABA is non-functional (Loveys 1977; Georgopoulou & Milborrow 2012). However, given the observation in an evergreen *Quercus* species and other herbaceous species, that chloroplast number is very low in young, expanding leaves, increasing as leaves expand (Miyazawa, Makino & Terashima 2003), this possibility seems unlikely. The most likely explanation is that the high levels of ABA found in the expanding leaves of *Q. rubra* are responsible for keeping stomata closed as leaves expand, although given other signals can close

stomata (Granot, Kelly, Stein & David-Schwartz 2014; Salmon *et al.* 2020) more experimental work is required to test this theory.

Based on this work the apparent order of events in expanding *Q. rubra* leaves is that very young leaves have relatively high levels of cuticular water loss that decline as leaves cease expanding. During expansion, stomata develop, but are present in low numbers and covered with a cuticle. Foliage ABA levels are initially high, and decrease through time as leaves expand, possibly keeping the youngest stomata closed under the cuticle, until the cuticle connecting the guard cells tears to form the stomatal aperture or is torn open by the opening stomata. Once the outer cuticular ledge forms, stomata accounts for most of the water lost from expanded leaves. This chain of events is very different to the model proposed by Pantin *et al.* (2013) based on observations made in *A. thaliana*. We would argue that these differences are not due to differences in species, as we found similar morphological development in the expanding leaves of both *Quercus* and *Arabidopsis*. However, further work is required to investigate the importance of cuticular conductance in leaf gas exchange as leaves expand across a wide diversity of species and also under field conditions. We find that the Pantin *et al.* (2013) model is not supported by our observations of very high levels of ABA measured in young leaves, the cuticle covering of young stomata, and the relatively late development of the outer cuticular ledge in expanding leaves of *A. thaliana* and *Q. rubra* all of which run counter to the theory that stomata are wide open and responsible for all of the water loss from young, expanding leaves. We conclude that the cuticle plays a primary role in determining the rate of water loss from expanding leaves.

## 2.6 Acknowledgments

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## CHAPTER 3. ABSISIC ACID DRIVEN STOMATAL CLOSURE DURING DROUGHT IN ANISOHYDRIC *FAGUS SYLVATICA*

The text and results of this chapter are taken directly from the following publication:

Kane, C., & McAdam, S. (2023). Absciscic acid driven stomatal closure during drought in anisohydric *Fagus sylvatica*. *Journal of Plant Hydraulics*, 9, 002-002.

### 3.1 Abstract

Stomatal closure limits transpiration during drought, restricting water potential decline and delaying the onset of embolism. While critical for ensuring survival during drought, the mechanisms driving stomatal closure during drought remain equivocal. The hormone abscisic acid (ABA) will close stomata in seed plants and is synthesized as leaf turgor declines. ABA driven stomatal closure during drought is particularly apparent in species that are more isohydric. In contrast, in species that have a more anisohydric response to drought, like *Fagus sylvatica*, the importance of ABA in driving stomatal closure during drought is often overlooked or excluded, in place of a hypothesized passive, water potential driven stomatal closure. Here we track ABA levels, leaf water potential and stomatal conductance during a natural summer drought in an individual of *F. sylvatica*. As leaf water potential declines to within 0.3 MPa of turgor loss point during a drought, foliage abscisic acid (ABA) levels increase considerably and stomata close. Foliage ABA levels correlate with stomatal conductance throughout a drought and post-drought period. These observations make it hard to exclude increased ABA levels as a driving mechanism for stomatal closure during drought in *F. sylvatica*.

### 3.2 Introduction

Drought is an increasing risk for global forests as climate change increases the frequency and severity of droughts (Allen *et al.* 2010; Adams *et al.* 2017; Martinez del Castillo *et al.* 2022). Climate change is already causing increased tree mortality, including in beech forests (Geßler *et al.* 2007; Allen *et al.* 2010; Anderegg, Anderegg, Abatzoglou, Hausladen & Berry 2013; Senf, Buras, Zang, Rammig & Seidl 2020; Hartmann *et al.* 2022). When plants experience long-term rainfall deficit soil water potential declines and water in the xylem experiences increasingly negative tension until a critical threshold is breached and the adhesion and cohesion forces can no longer hold the column of water



together causing an embolism in the xylem tissue (Tyree & Sperry 1989; Sperry & Pockman 1993; Brodribb & Hill 1999; Cardoso, Kane, Rimer & McAdam 2022). Embolism is irreversible in most cases and if sufficient embolism accumulates, hydraulic conductivity cannot match residual transpiration at which point water potentials catastrophically decline leading to tissue damage and even whole plant death (Brodribb & Cochard 2009; Charrier *et al.* 2016; Cardoso, Batz & McAdam 2020a; Mantova *et al.* 2023). During periods of water deficit plants are able to limit evaporation quickly by closing stomata, which reduces declines in water potential, limiting the chance of embolism during drought (Brodribb & McAdam 2013; Martin-StPaul, Delzon & Cochard 2017; Lamarque *et al.* 2018; Brodribb *et al.* 2021).

One strategy that allows plants to occupy seasonally dry environments is the evolution of highly embolism resistant xylem that can transport liquid water at very negative water potentials ( $< -5$  MPa) without experiencing embolism (Larter *et al.* 2017; Skelton, Brodribb, McAdam & Mitchell 2017). The building of highly embolism resistant xylem is not the only strategy that allows plants to live in environments that experience a seasonal drought, some species have evolved the capacity to greatly reduce transpiration, by very effectively closing stomata and having very low cuticular conductance which minimizes residual transpiration to such an extent that water potentials decline very slowly under drought conditions (Klein 2014; Duursma *et al.* 2019; Kane, Jordan, Jansen & McAdam 2020). These two very different strategies to survive drought form the basis of the isohydric/anisohydric continuum (Jones 1998; Tardieu & Simonneau 1998; McDowell *et al.* 2008; Leuschner, Schipka & Backes 2022). Species classified as more isohydric tend to have stomata that are more effective at closing in response to drought, consequently they often have more vulnerable xylem, and can disconnect plant water potential from soil water potential very effectively during drought. More anisohydric species tend to have stomata that are less responsive to declines in water potential, more embolism resistant xylem, smaller hydraulic safety margins, and a stronger relationship between soil and plant water potential during drought (Jones 1998; Tardieu & Simonneau 1998; McDowell *et al.* 2008; Sade, Gebremedhin & Moshelion 2012; Attia, Domec, Oren, Way & Moshelion 2015; Martínez-Vilalta & Garcia-Forner 2017; Leuschner *et al.* 2022). The isohydric/anisohydric distinction can be viewed as a spectrum rather than two categories that all land plants will fit neatly into (Klein 2014; Martínez-Vilalta & Garcia-Forner 2017).

European beech *Fagus sylvatica* L. is classically characterized as an anisohydric species (Pretzsch *et al.* 2014; Leuschner *et al.* 2022; Hesse *et al.* 2022), although the degree of isohydricity in

this species appears to be flexible across rainfall gradients with populations originating from drier environments exhibiting more isohydric stomatal control while plants from wetter regions having more anisohydric stomatal responses to water potential (Nguyen 2016; Nguyen, Polle & Pena 2017). Leuschner *et al* (2022) determined that by most metrics, including the relationship between leaf and soil water potential and the size of the hydroscape that *F. sylvatica* should be classed as an anisohydric species, while their data on the relationship between leaf water potential and leaf stomatal conductance indicate a slightly more isohydric behavior that may shift throughout the season from more isohydric to more anisohydric and that the stomata of *F. sylvatica* are very sensitive to declining leaf water potential and changes in vapor pressure deficit. Leuschner *et al* (2022) states that a root borne signal is the most likely driver of stomatal closure during seasonal drought in *F. sylvatica* and speculates that this signal may be a hormone but stops short at implicating abscisic acid (ABA).

ABA is a dynamic plant hormone well known to close stomata during drought in seed plants (Beardsell and Cohen, 1975; McAdam *et al.*, 2016; Munemasa *et al.*, 2015; Mustilli *et al.*, 2002; Nguyen, 2016; Susmilch *et al.*, 2017a). ABA levels rise in leaf tissues as water potentials decline (Wright 1977) and in response to VPD (McAdam *et al.* 2016; Cardoso, Brodribb, Kane, DaMatta & McAdam 2020b). ABA is primarily synthesized in the leaf mesophyll and not the roots as was once suspected (Holbrook, Shashidhar, James & Munns 2002; McAdam & Brodribb 2016, 2018; Zhang *et al.* 2018). As leaves dry ABA is produced once mesophyll cells lose turgor presumably because of changes to a critical membrane-cell wall interaction (Bacete *et al.*, 2022; Cardoso *et al.*, 2020b; McAdam and Brodribb, 2016; Susmilch *et al.*, 2017b). ABA production during drought has been shown to differ between anisohydric and isohydric species, with isohydric species relying on ABA to close stomata during drought, while some anisohydric exhibiting a switch from having stomata controlled by ABA to water potential driven stomatal closure on repeated droughts and rewatering (Tardieu & Simonneau 1998; Nolan *et al.* 2017). Or in some cases supposedly not requiring ABA to close stomata during drought at all (Tardieu & Simonneau 1998; Thomas, Eamus & Shanahan 2000; Torres-Ruiz, Diaz-Espejo, Perez-Martin & Hernandez-Santana 2015).

Here we sought to document potential relationships between ABA level and gas exchange during a natural drought in a repeatedly-sampled individual of *F. sylvatica*. We tracked leaf gas exchange, leaf ABA level, leaf water potential in a specimen tree of *F. sylvatica* f. *purpurea* during a natural drought in West Lafayette, IN, USA in the summer of 2021. In addition, we measured

the turgor loss point of leaves from the same tree to determine if there was a relationship between the water potential at which ABA is produced in the field and when leaves lose turgor. *F. sylvatica* was selected due to its importance in European forests, the imminent threat climate change poses to this species (Knoke, Stang, Remler & Seifert 2006; Geßler *et al.* 2007), and the equivocal role of hormones in stomatal regulation in this species (Leuschner *et al.* 2022).

### 3.3 Methods

#### 3.3.1 Plant material

A mature specimen tree of *F. sylvatica* f. *purpurea* L. (Fagaceae) growing on the Purdue University campus in West Lafayette, Indiana, USA (40.425224, -86.914636) was selected for these observations. *F. sylvatica* f. *purpurea* is a cosmopolitan ornamental tree with a naturally occurring mutation that makes the leaves appear red or purple (Nonić, Skočajić, Grbić & Šijačić-Nikolić 2017). All individuals in cultivation are clones of a single, now extinct, tree called Mutterblutbuche that arose naturally in the Heinleite hills south of Sondershausen in the German state of Thuringia (Lutze 1892; Sargent 1894). The site of origin of this cultivar receives 600-700mm of precipitation annually ([www.dwd.de](http://www.dwd.de)) which would mean that this individual is most likely anisohydric based on the work of Nguyen *et al.* (2017), who observed more isohydric individuals native only to regions that received less than 550 mm of rainfall per year. The individual in this study was grown under higher annual precipitation with West Lafayette, Tippecanoe County, IN, USA receiving approximately 900mm per year ([www.noaa.gov](http://www.noaa.gov)).

#### 3.3.2 Data collection

Three leaves were measured on six campaigns spread over the course of 93 days from summer (July) to autumn (October) 2021. Gas exchange was measured on sunny days in three, attached, sun exposed leaves using an infrared gas analyzer (LI-6800 Portable Photosynthesis System; LI-COR Biosciences, Lincoln, NE, USA). The conditions in the cuvette were set to ambient CO<sub>2</sub>, ambient VPD and a saturating light intensity of 1500  $\mu\text{mol m}^{-2} \text{s}^{-1}$  to measure instantaneous CO<sub>2</sub> assimilation and stomatal conductance. Immediately following gas exchange measurements leaves were excised, wrapped in damp paper towel, and enclosed in plastic zip top bag to halt transpiration. Leaves were then taken back to the lab where leaf water potentials were

measured using a Scholander pressure chamber (PMS Instrument Company, OR, USA) with microscope. After slow depressurization of the Scholander chamber, leaf tissue was harvested into a tube to measure leaf ABA content.

Leaf ABA content was measured by physicochemical methods with an added internal standard according to McAdam (2015). The mass of the fresh foliage sample was recorded on an analytical balance ( $\pm 0.0001$  g, OHAUS Corporation, Parsippany, NJ, USA) where fresh leaf tissue was placed into a 25 ml tube and completely covered in  $-20^{\circ}\text{C}$  acetone containing  $250\text{ mg l}^{-1}$  butylated hydroxytoluene. The leaf tissue was then chopped into fine pieces and stored in a  $-20^{\circ}\text{C}$  freezer overnight. The leaf tissue was homogenized and  $15\text{ ng}$  of  $[^2\text{H}_5]\text{ABA}$  was added to each sample as an internal standard before extracting overnight at  $4^{\circ}\text{C}$ . An aliquot of supernatant, approximately  $2\text{ ml}$ , was taken from each sample and dried down in a vacuum sample concentrator (Labconco, MO, USA), the ABA and internal standard were then resuspended in  $200\text{ }\mu\text{l}$  of 2% acetic acid in water ( $v\text{ v}^{-1}$ ), after which it was centrifuged at  $14,800\text{ RPM}$  for 4 minutes.  $100\text{ }\mu\text{l}$  was taken for quantification of ABA and internal standard levels using an Agilent 6460 series triple quadrupole LC/MS (Agilent, CA, USA). After quantification, the homogenized leaf samples were dried at  $70^{\circ}\text{C}$ , and leaf dry mass was determined by subtracting the mass of the clean empty tube from the mass of the tube containing the dried homogenized leaf material.

Leaf chlorophyll *a* content was measured from the same sample as ABA after extraction in  $-20^{\circ}\text{C}$  acetone containing  $250\text{ mg l}^{-1}$  butylated hydroxytoluene.  $5\text{ }\mu\text{l}$  of the liquid from each sample was placed onto a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific, MA, USA) pedestal and absorbance at  $433\text{ nm}$  was measured this was then quantified using a standard curve (ThermoFisher Scientific 2018). The total volume of acetone in the sample was determined gravimetrically by obtaining the mass of the total homogenized sample in acetone, then drying the sample at  $70^{\circ}\text{C}$  and determining dry mass of the vial and sample. The volume of acetone was determined by multiplying the mass change and the density of acetone at standard atmospheric pressure at  $180\text{ m}$  above sea level and adding back the  $2\text{ ml}$  taken for ABA analysis and the  $5\text{ }\mu\text{l}$  take for spectrophotometry. Dry mass of the sample was then determined by cleaning the tube and redrying the vial to determine the mass of the clean tube which was subtracted from the mass of the dry sample with no acetone (Kane & McAdam 2023).

Turgor loss point was determined by pressure-volume curves (Tyree & Hammel 1972). Unstressed leaves were wrapped in damp paper towel, bagged in plastic, and rehydrated overnight

by recutting the petiole under deionized water in a shallow glass dish, being sure to keep the bag, towel, and lamina out of the water. The leaves were then measured in a Scholander pressure chamber (PMS Instrument Company, OR, USA) and immediately moved onto an analytical balance and the mass recorded ( $\pm 0.0001$  g, OHAUS Corporation, Parsippany, NJ, USA). This was repeated 9 times with 6 leaves over the course of 6 hours as leaves dehydrated slowly on the bench. The leaves were then scanned for leaf area using an Epson Perfection V39 Flatbed Scanner (EPSON, Japan). The leaves were then dried at 60°C for at least 72 h and dry weight taken. The curves were analyzed using the PVASt spreadsheet according to (Bartlett, Scoffoni & Sack 2012).

Rain fall data were collected from the Purdue University Weather station at the Purdue Airport (LAF) located around 2 km from the field site (wunderground.com). Drought status was determined from the U.S. Drought Monitor (droughtmonitor.unl.edu) which uses data from NOAA and NIDIS.

### **3.3.3 Data analysis**

Exponential decay single 2 parameter regressions were fit on to the raw data for foliage ABA levels, stomatal conductance, and leaf water potential using SigmaPlot 10.0 (SYSTAT, CA, USA). A linear regression was fit to foliage ABA vs leaf water potential using SigmaPlot 10.0 (SYSTAT, CA, USA). All time course data was analyzed using a one-way ANOVA and post hoc Tukey's test. All time-course data are plotted against days since winter solstice which allows for a standardized comparison of phenological data for readers in both northern and southern hemispheres. Water use efficiency was calculated as the quotient of CO<sub>2</sub> assimilation rate by stomatal conductance for each leaf measured.

Table 3-1. The collection dates as days since winter solstice (DAWS) and day of year (DOY). Accompanying each date is the mean and standard errors (n=3) of leaf water potential, foliage ABA level, leaf chlorophyll *a* content, stomatal conductance, photosynthetic rate and water use efficiency (WUE)

DAWS	DOY	Leaf water potential (MPa)	Foliage ABA level (ng g <sup>-1</sup> DW)	Leaf chlorophyll <i>a</i> content (mg g <sup>-1</sup> DW)	Stomatal conductance (mol m <sup>-2</sup> s <sup>-1</sup> )	A <sub>sat</sub> (μmol CO <sub>2</sub> m <sup>-2</sup> s <sup>-1</sup> )	WUE (μmol mol <sup>-1</sup> )
210	200	-0.96 ± 0.26	47.2 ± 2.7	7.1 ± 0.61	0.22 ± 0.02	14 ± 0.98	62.6 ± 5.5
237	227	-1.93 ± 0.09	94.4 ± 12.1	7.09 ± 0.23	0.056 ± 0.01	6.47 ± 1.3	114 ± 1
255	245	-1.77 ± 0.04	136 ± 37.3	7.65 ± 0.88	0.03 ± 0.0062	4.94 ± 0.52	132 ± 7.6
271	261	-1.73 ± 0.08	64.1 ± 13.1	6.87 ± 0.53	0.04 ± 0.01	5.57 ± 0.97	123 ± 6.7
283	273	-0.9 ± 0.06	54.6 ± 10.2	5.54 ± 0.42	0.1 ± 0.001	9.88 ± 0.13	97.4 ± 1.5
303	293	-0.76 ± 0.02	45.2 ± 2.7	4.88 ± 0.05	0.1 ± 0.012	7.70 ± 0.55	78.4 ± 13.9

### 3.4 Results

During the summer of 2021 Tippecanoe county, IN, experienced an abnormally dry period (D0 –which is the mildest drought category used by the U.S. Drought Monitor, it is based on low percentile values for a combination of key indices including soil moisture levels and local stream flow dropping into the lowest 20<sup>th</sup> percentile of historic records, and a standardized precipitation index below -0.5), this period lasted from summer into the early autumn 232-281 days after winter solstice (DAWS) (Figure 3-1A insert). Prior to the onset of this dry period levels of leaf ABA were low at  $47.28 \pm 2.69$  ng g<sup>-1</sup> at 210 DAWS (Figure 3-1A insert) this corresponded to the time point when we measured the highest rates of stomatal conductance during this study at  $0.227 \pm 0.027$  mol m<sup>-2</sup> s<sup>-1</sup> (Figure 3-1B) and an average leaf water potential of  $-0.96 \pm 0.27$  MPa (Figure 3-1C) these are both significantly higher than the subsequent 3 measurements which took place during the dry period. Leaves measured at 237 DAWS, 5 days into the abnormally dry period and 30 days since at least 20 mm of rain had fallen, had higher levels of leaf ABA content, with mean levels doubling (Figure 1A) and a mean leaf water potential declining to  $-1.93 \pm 0.09$  MPa (Figure 3-1C) and stomatal conductance of  $0.057 \pm 0.012$  mol m<sup>-2</sup>s<sup>-1</sup> (Figure 3-1B). The highest levels of leaf ABA were measured 255 DAWS, 22 days into the abnormally dry period, when levels had

increased to  $136.53 \pm 37.39 \text{ ng g}^{-1}$  (Figure 3-1A) this corresponded with the lowest rates of stomatal conductance measured during the study period at  $0.038 \pm 0.006 \text{ mol m}^{-2}\text{s}^{-1}$  (Figure 3-1B) both measurements being significantly different from the prestress measurements. 271 DAWS 10 days before the end of the abnormally dry period, and 3 days after a brief rainfall event of 3.05 mm, the three measured leaves were variable with one leaf recording a leaf ABA content of  $39.96 \text{ ng g}^{-1}$  (Figure 3-1A red circle) this leaf had the highest rate of stomatal conductance and leaf water potential measured at this time point, at  $0.066 \text{ mol m}^{-2}\text{s}^{-1}$  (Figure 3-1B red circle) and  $-1.53 \text{ MPa}$  (Figure 3-1C red circle), respectively. The other two leaves measured on this day had higher levels of leaf ABA content and more negative water potentials which corresponded with lower rates of stomatal conductance (Figure 3-1A, B, C white circles). After the dry period ended at 281 DAWS the next leaves collected on 283 DAWS and 303 DAWS showed reduced leaf ABA content, less negative water potentials, and higher stomatal conductance relative to the dry period. By 303 DAWS leaf ABA levels had fallen to the lowest measured during the study period at an average of  $45.26 \pm 2.78 \text{ ng g}^{-1}$  (Figure 3-1A), this corresponded with the lowest average water potential measured during the study period  $-0.77 \pm 0.03 \text{ MPa}$ , yet stomatal conductance did not recover to the maximum rates measured in early summer.  $\text{CO}_2$  assimilation ( $A_{\text{sat}}$ ) rate through the dry period was like that of stomatal conductance declining from a maximum of  $14.03 \pm 0.98 \mu\text{mol CO}_2 \text{ m}^{-2}\text{s}^{-1}$  210 DAWS to a low of  $4.95 \pm 0.53 \mu\text{mol CO}_2 \text{ m}^{-2}\text{s}^{-1}$  255 DAWS (Table 3-1).  $A_{\text{sat}}$  did not return to predrought levels after the end of the dry period only returning to  $9.88 \pm 0.14 \mu\text{mol CO}_2 \text{ m}^{-2}\text{s}^{-1}$  283 DAWS (Table 3-1). An increase in water use efficiency was observed during the dry period increasing significantly from  $62 \pm 5.5 \mu\text{mol mol}^{-1}$  at 210 DAWS to  $114 \pm 1 \mu\text{mol mol}^{-1}$  at 237 DAWS only 5 days after the onset of the abnormally dry period. After rainfall WUE returned to a similar pre-drought level despite stomata not reopening to maximum apertures (Figure 3-1B insert). Mean leaf chlorophyll *a* content was stable for the duration of the dry period with levels around  $7.11 \pm 0.61 \text{ mg g}^{-1}$  at 210 DAWS but once the dry period ended chlorophyll *a* content began declining to  $5.54 \pm 0.43 \text{ mg g}^{-1} \text{ DW}$  at 283 DAWS two days after the end of the dry period then to  $4.89 \pm 0.05 \text{ mg g}^{-1} \text{ DW}$  28 days after the autumn equinox at 303 DAWS (Figure 3-1D).

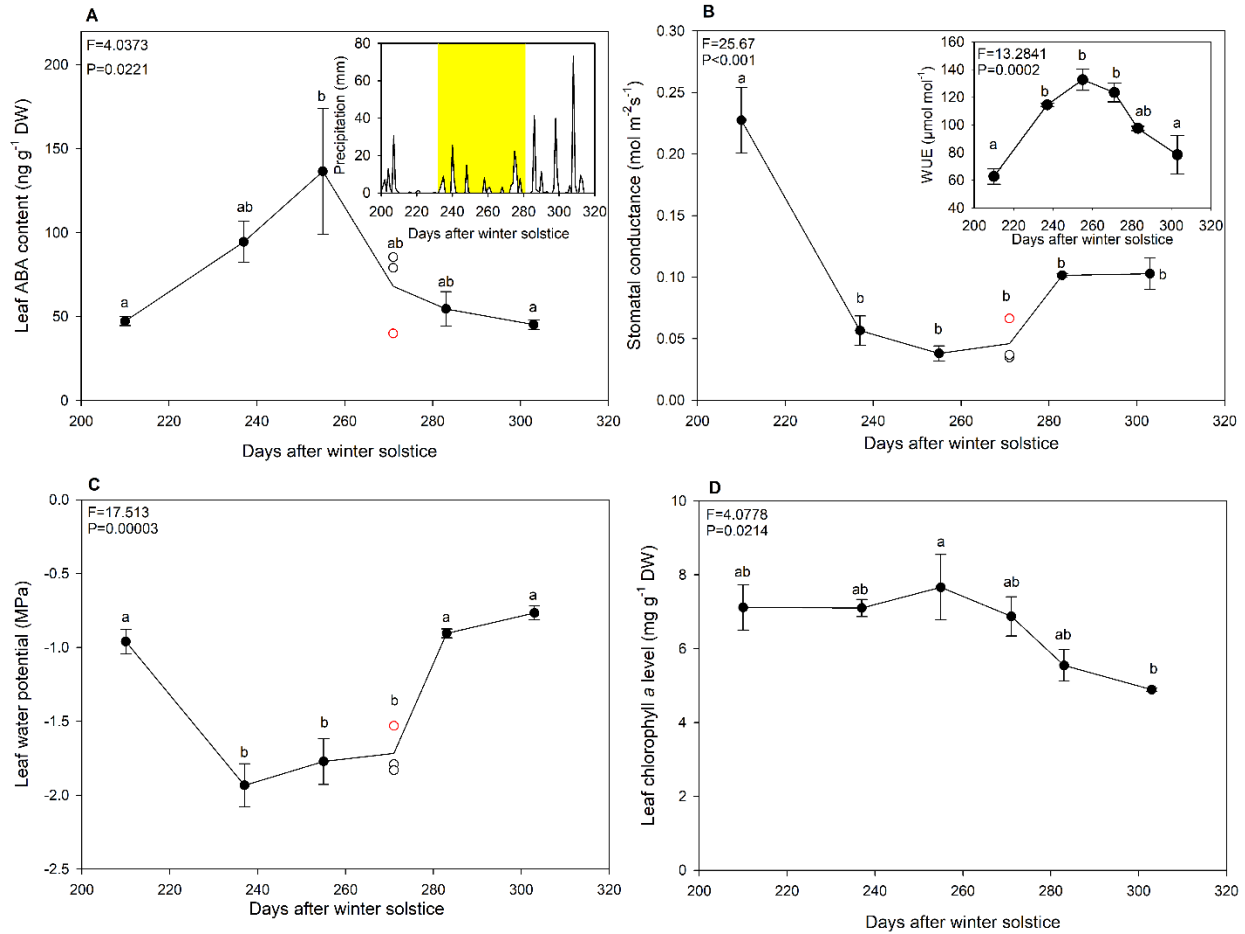


Figure 3-1. (A) Mean leaf ABA content, (B) stomatal conductance, (C) leaf water potential, (D) leaf chlorophyll *a* content through a natural drought in an individual of *Fagus sylvatica* f. *purpurea* in West Lafayette, Indiana, USA. Each black point represents an average of 3 sun exposed leaves measured at midday and are accompanied with standard error bars. The two white and one red point on 271 days after winter solstice show the individual values of leaf ABA content, stomatal conductance, leaf water potential for each leaf collected on that day with the trend line passing through the average of those points. The insert in A depicts the rain fall during the study period with the yellow area indicating the abnormally dry period (D0). The insert in (B) depicts water use efficiency through the drought. One-way ANOVAs were performed on all time course data and the F-statistic and P value are displayed, Tukey's post hoc tests were performed, and significant differences are denoted by different letters.

Stomatal conductance and leaf ABA content in *F. sylvatica* formed a significant ( $P=0.0084$ ;  $R^2=0.3611$ ) exponential decay relationship ( $\text{Stomata conductance} = 0.3122 * e^{(-0.0187 * \text{Leaf ABA content})}$ ), with stomatal conductance exponentially declining as leaf ABA content increased (Figure 3-2A). We also observed a significant linear relationship ( $P=0.0033$ ;  $R^2=0.4621$ ) between stomatal conductance and leaf water potential ( $\text{Stomatal conductance} = 0.212 + 0.0869 * \text{Leaf water potential}$ ).



Similarly, we found a significant ( $P=0.0066$ ;  $R^2=0.3785$ ) exponential decay relationship ( $\text{Leaf ABA content} = 28.1118 * e^{(-0.6836 * \text{Leaf water potential})}$ ) between leaf ABA content and leaf water potential over the year in *F. sylvatica*. The highest levels of leaf ABA measured during the study period ( $>75 \text{ ng g}^{-1}$ ) were measured only when leaves were within  $\pm 0.34 \text{ MPa}$  of the average leaf turgor loss point of  $-2.02 \pm 0.09 \text{ MPa}$  (Figure 3-2B).

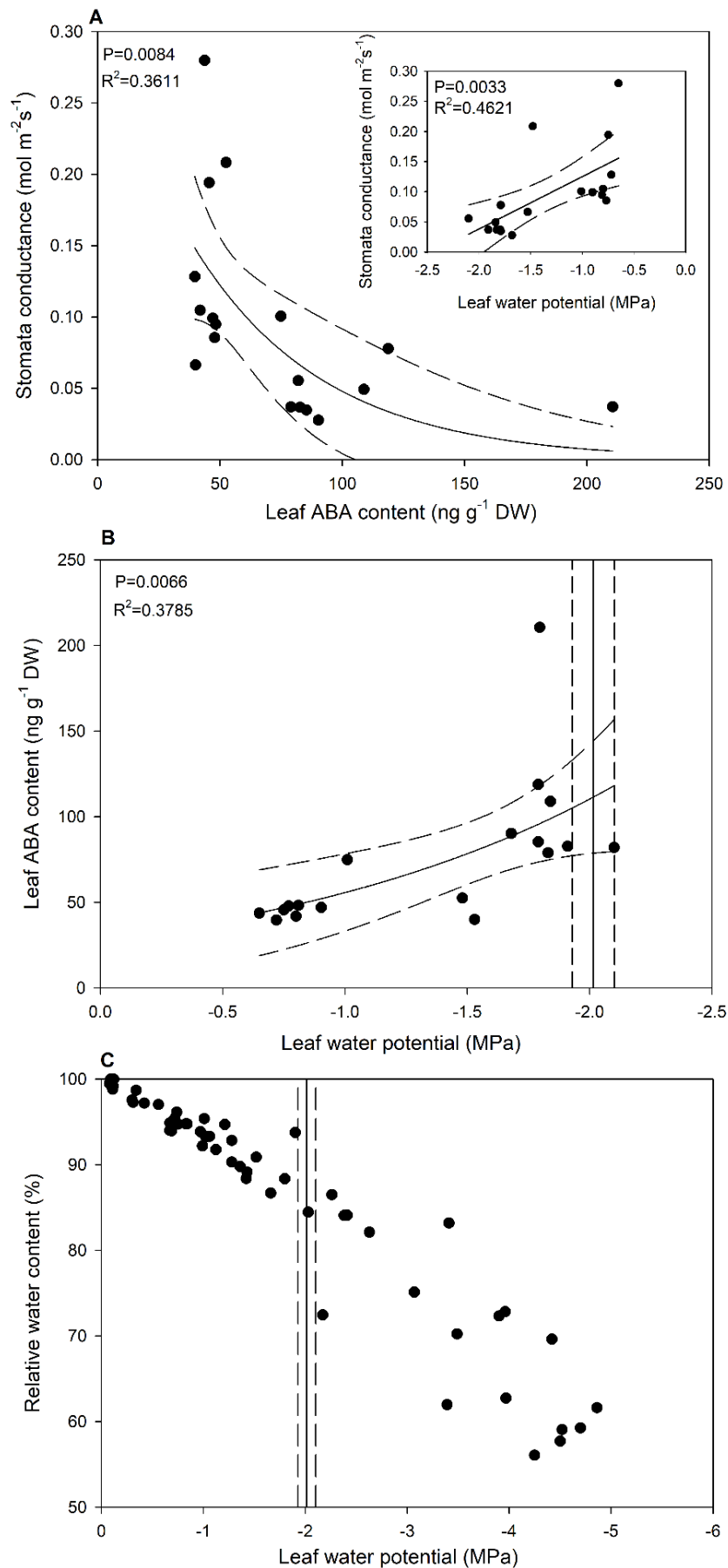


Figure 3-2. (A) The relationship between leaf ABA content and stomatal conductance and (B) leaf water potential and leaf ABA content for leaves sampled through a natural drought in West Lafayette, Indiana, USA in 2021. Each point represents one sun leaf collected during midday from an individual *Fagus sylvatica f. purpurea*. Exponential decay regressions are fitted to the data with dashed lines representing 95% confidence intervals. The vertical solid line in B depicts the mean turgor loss point of 6 individual sun leaves from the same individual with the dashed vertical lines showing the standard error of the turgor loss point. Insert in (A) shows the linear relationship between stomatal conductance and leaf water potential. (C) Pressure-volume curve for leaves of *Fagus sylvatica f. purpurea*. Each point represents one measurement of one of the 6 leaves with each leaf being measured 9 times during the dry down. The vertical solid line shows the mean turgor loss point of 6 individual sun leaves from the same individual with the dashed vertical lines showing the standard error of the turgor loss point.

### 3.5 Discussion

We find that while *F. sylvatica* which is traditionally classed as an anisohydric species (Pretzsch *et al.* 2014; Leuschner *et al.* 2022; Hesse *et al.* 2022) does not mean that ABA plays no role in driving stomatal closure during drought. Our results suggest that water potential and turgor driven production of the drought stress hormone ABA might drive stomatal closure in *F. sylvatica* during drought. Our data support the conclusion of Leuschner *et al.* (2022) that stomatal control in *F. sylvatica* is sensitive to declining leaf water potential, but we show that this relationship may be mediated by ABA because as leaf water potentials become more negative and stomatal conductance declines, leaf ABA levels rises. We observed significant relationships between stomatal conductance and both forces that could potentially close stomata including increasing ABA levels and declining leaf water potential. While our observations make it impossible to separate the effect of ABA and leaf water potential on stomatal conductance during drought it is tempting to speculate that an increase in ABA level closes stomata initially during drought and that this stomatal closure is facilitated by further declines in leaf turgor, especially if the epidermal pavement cells lose turgor (Rodriguez-Dominguez *et al.* 2016; Buckley 2019).

Our observations, while limited to a single individual, suggest that ABA driven stomatal closure during drought occurs before embolism induced declines in hydraulic conductivity, or even incipient embolism formation. Published embolism resistance data for *F. sylvatica* suggest that P50 ranges from -1.5 to -3.94 MPa depending on the region of origin as well as position in the canopy (Guan *et al.* 2022; Lemonie *et al.* 2002). Hacke and Sauter (1995) found in individuals of *F. sylvatica* f. *purpurea* in Northern Germany that embolism begins to occur in this variety at a water potential more negative than -1.9 MPa with stem P50 occurring around -2.9 MPa. Hacke and Sauter (1995) suggest that in the summer plants of *F. sylvatica* f. *purpurea* rarely experience water potentials more negative than -1.9 MPa which matches our observations here. During our study period we only observed two leaves with water potentials less than -1.9 MPa both observed on the same day (237 DAWS) during the abnormally dry period. This same day we saw stomatal conductance having declined by more than 75% of that measured prior to the drought (210 DAWS) and these leaves had double the average leaf ABA content. In other work in *F. sylvatica* at Southern European sites with the lowest annual rainfall across the species range daily leaf water potentials rarely fall below -2 MPa and only in upper canopy leaves (Aranda, Gil & Pardos 2000). These and our observations support the conclusions of Guan *et al.* (2022) and Leuschner (2020)

that there is a considerable safety margin between the water potential of stomatal closure and P50 in *F. sylvatica*.

ABA has long been known to close stomata (Mittelheuser & Van Steveninck 1969; Beardsell & Cohen 1975) and this link is understood to be associated with driving stomatal closure during drought in seed plants (Wright 1977). While limited, our observations during a drought suggest that this might also be the case for *F. sylvatica f. purpurea*. Nguyen (2016) observed that *F. sylvatica* plants grown in drier conditions showed higher expression of ABA related stress genes and lower water loss rates but did not measure ABA levels. Leuschner *et al* (2022) speculates that there could be a hormonal control of stomatal closure in *F. sylvatica* but preferred the explanation that a lasting loss of bulk leaf turgor passively closed stomata as the most plausible explanation for the long term (May to August) partial stomatal closure observed in the field when leaf water potential declined between -1 to -1.5 MPa. Our observations cannot rule out the possibility that like many other seed plant species ABA, synthesized by leaves near turgor loss point, is closing stomata under these conditions in *F. sylvatica*. Leuschner *et al.* (2022) also speculates that the signal for stomatal closure originates in the roots because isohydric species can maintain a stable leaf water potential despite soil drying. Modern work has shown that ABA is primarily produced in leaf tissue during periods of water deficit (Holbrook *et al.* 2002; McAdam & Brodribb 2016, 2018; Zhang *et al.* 2018), and that the signal is driven by a loss of cell turgor. Our results along with the observations of Hacke and Sauter (1995) suggest that this might also be the case in *F. sylvatica*. The embolism onset threshold of -1.9 MPa observed by Hacke and Sauter (1995) in this clone also corresponds closely to the turgor loss point we measured in leaves of *F. sylvatica f. purpurea* which was  $-2.02 \pm 0.09$  MPa the lower bound being -1.93 MPa indicating that turgor loss point may be an important drought threshold for *F. sylvatica f. purpurea*. Leuschner *et al* (2022) questioned whether a hormonal signal like ABA could increase and keep stomata closed for a sustained period ‘weeks or months’, we show here in this single-season study that elevated levels of leaf ABA can be observed during drought in an individual of this species for least 34 days. There are many other reports of sustained high levels of ABA in leaves during long-term drought (Thomas *et al.* 2000; Wang, Mopper & Hasenstein 2001; Brodribb, McAdam, Jordan & Martins 2014; Tombesi *et al.* 2015). Based on the relationships we observed between ABA level, leaf water potential and stomatal conductance, we cannot exclude an influence of leaf water potential acting directly on stomatal conductance in *F. sylvatica* to drive stomatal closure during drought. It could be that ABA and leaf

water potential act in concert to close and maintain the closure of stomata during drought, although this speculation will need further study to resolve and in *F. sylvatica* might also be dependent on the geographic origin of the individual (Nguyen *et al.* 2017).

We observed that upon the end of the dry period in 2021 leaf ABA content and leaf water potential recovered to predrought levels, but that even though stomatal conductance increased gas exchange did not return to the levels measured prior to the drought. We believe that there are two possible reasons for this that are not mutually exclusive. Based on the observations of Hacke and Sauter (1995) leaves may have reached water potentials that could cause some low levels of embolism which can reduce maximum stomatal conductance upon recovery (Skelton *et al.* 2017; Cardoso *et al.* 2020a). Given that midday water potentials recovered to predrought levels in our study we believe that it is unlikely that stomatal opening was suppressed by embolism-induced declines in hydraulic conductance. The other, more likely, explanation is that stomatal conductance may be limited by the seasonal phenology of this deciduous species (Abadía *et al.* 1996; Wang, Gao & Ge 2022). The first measurement after the end of the drought (281 DAWS) occurred on 283 DAWS (September 30, 2021), more than 100 days after the summer solstice and a week after the autumn equinox in the Northern Hemisphere. We observed a 31% reduction in average chlorophyll *a* content in leaves of *F. sylvatica* *f. purpurea* between the initial and final measurement of this study, which likely drove lower assimilation rates and may have been hastened by the high levels of leaf ABA induced by the drought as observed in other deciduous species (Kane & McAdam 2023). These may have acted independently or in concert with the drought to accelerate leaf senescence (Thimann & Satler 1979b a; Radin 1981). It is not yet settled if drought influences senescence rate in *F. sylvatica* (Mariën *et al.* 2021; Frei *et al.* 2022).

The decrease in leaf water potential and increase in ABA levels, driving stomatal closure during the dry period, caused a two-fold increase in WUE in this individual (Figure 3.1). This increase suggests a more dramatic influence of ABA or low water potential on stomatal conductance during drought relative to photosynthetic rate, which improves the optimization of water use (Dubbe, Farquhar & Raschke 1978). When ABA levels declined and leaf water potential recovered after the dry period, WUE declined to pre-drought levels, this occurred even though stomata did not open to maximum rates of conductance – which we attribute to autumnal declines in chlorophyll content lowering maximum photosynthetic rates (Figure 3.1). Our results suggest that declines in stomatal closure during drought have a more profound influence of WUE than

declines in chlorophyll content and the associated reduction in maximum photosynthetic rate during senescence. The feedback of lower photosynthesis on stomatal conductance in the autumn as chlorophyll content declines did not improve WUE.

During the abnormally dry period of 2021 in West Lafayette, Indiana, we observed more negative water potentials, reduced stomatal conductance, and higher levels of leaf ABA content in an individual of *F. sylvatica*. Before and after the dry period leaves were at higher water potentials, had higher rates of stomatal conductance, and lower levels of leaf ABA content. The results of this study provides additional evidence that the iso/anisohydric classification may be more correctly viewed as a spectrum made up of a collection of traits rather than two distinct categories (Jones 1998; Tardieu & Simonneau 1998; McDowell *et al.* 2008; Sade & Moshelion 2014; Chen *et al.* 2021; Bryant, Fredericksen & Rosenthal 2022; Leuschner *et al.* 2022). This is especially true in species like *F. sylvatica* which appear to have considerable plasticity in traits that are used for classification as iso/anisohydric depending on the rates of precipitation the provenance receives or the time of year samples are taken (Nguyen 2016; Leuschner 2020; Leuschner *et al.* 2022). Our descriptive observations provide several hypotheses that should be tested in future studies, including whether ABA deficiency in *F. sylvatica* results in a wilting phenotype, or if there is variation across the range of *F. sylvatica* in the degree of hormonal control of stomata. Future studies could also address some of the critical limitations of this descriptive work, particularly those associated with a limited number of collections dates, use of a single individual, testing whether osmotic adjustment occurred during drought and lack of predawn water potentials which would all help to further resolve the role of hormonal control of stomata in *F. sylvatica*.

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## **CHAPTER 4. ABSCISIC ACID CAN AUGMENT, BUT IS NOT ESSENTIAL FOR, AUTUMNAL LEAF SENESCENCE**

The text and results of this chapter are taken directly from the following publication:

Kane, C. N., & McAdam, S. A. (2023). Absciscic acid can augment, but is not essential for, autumnal leaf senescence. *Journal of Experimental Botany*, 74(10), 3255-3266.

### **4.1 Abstract**

Senescence vividly marks the onset of the final stages of the life of a leaf, yet the triggers and drivers of this process are still not fully understood. The hormone abscisic acid (ABA) is an important regulator of leaf senescence in model herbs, but the function of this hormone has not been widely tested in deciduous trees. Here we investigate the importance of ABA as a driver of leaf senescence in winter deciduous trees. In four diverse species we tracked leaf gas exchange, water potential, chlorophyll content, and foliage ABA levels from the end of summer until leaves were abscised or died. We found that no change in ABA levels occurred at the onset of chlorophyll decline or throughout the duration of leaf senescence. To test whether ABA could enhance leaf senescence we girdled the branches to disrupt ABA export in the phloem. Girdling increased foliage ABA levels in two of the species, and this increase triggered an accelerated rate of chlorophyll decline in these species. We conclude that an increase in ABA level may augment leaf senescence in winter deciduous species but that it is not essential for this annual process.

### **4.2 Introduction**

Autumn leaf senescence is one of the final metabolic processes of the annual flush of leaves in deciduous temperate forests and annual crops, the timing of which is critical for determining leaf life span, through to influencing global carbon cycles and harvest time and post-harvest quality of agricultural products (Knorr 2000; Richardson *et al.* 2010, 2013). Senescence is important for the remobilization of nutrients from leaves to support new growth in the following spring (Lim, Kim & Gil Nam 2007), or to augment seed maturation (Lim *et al.* 2007). Despite the importance of this process, from carbon cycles to agriculture, there remains a suite of unpredictable and conflicting hypotheses explaining the physiological and molecular drivers of leaf senescence,

particularly in temperate deciduous tree species (Gan & Amasino 1997; Richardson *et al.* 2012; Liu *et al.* 2020). Temperate forests contribute around 30% of global forest carbon sequestration or around 11.1 metric tons of carbon per hectare per year, a process that happens almost exclusively in the spring and summer ending each autumn with the onset of leaf senescence (Wofsy *et al.* 1993; Pan *et al.* 2011). Consequently, being able to accurately model this process is critical for improving predictions of future biotic feedbacks on the changing global climate, yet this requires a comprehensive understanding of the mechanisms driving senescence (Zani, Crowther, Mo, Renner & Zohner 2020)

At the onset of autumnal leaf senescence, the leaf degrades chlorophyll leading to a charismatic seasonal leaf color change in many species (Lim *et al.* 2007), throughout this process the photosynthetic machinery is disassembled and lipids, proteins, and mobile nutrients are remobilized out of the leaf to be stored in the woody tissue (Smart 1994 420; Buchanan-Wollaston 1997). The known triggers and regulators of senescence are diverse, encompassing both internal and external stimuli including, leaf age, colder temperatures, shorter day length, shading, the onset of flowering or seed set and pathogen attack (Smart 1994; Noodén, Guimét & John 1997; Buchanan-Wollaston 1997; Woo, Kim, Lim & Nam 2019). The pathway through which plants sense and signal these drivers has not been fully elucidated, but phytohormones are believed to play a vital, coordinating role in translating the diverse environmental and endogenous cues into an initialization of the cascade of metabolic events defining senescence.

Ethylene is well established as a fundamental phytohormone responsible for triggering and accelerating leaf senescence (Aharoni & Lieberman 1979; Iqbal *et al.* 2017; Jibran *et al.* 2013). Absciscic acid (ABA), a hormone primarily associated with drought response, has long been thought of as another important phytohormone driving leaf senescence (Nooden & Leopold, 1988). ABA triggers and accelerates the rate of leaf senescence in leaf discs and detached leaves (El-Antably, Wareing & Hillman 1967; Chin & Beevers 1970; Gepstein & Thimann 1980; Philosoph-Hadas, Hadas & Aharoni 1993; Fan, Zheng & Wang 1997; Hung & Kao 2003; Gao *et al.* 2016; Zakari, Asad, Han, Zhao & Cheng 2020). It may also be involved in the lethal action of auxin-based herbicides (Grossmann 2010; McCauley *et al.* 2020). Yet the role of endogenous ABA in leaf senescence in intact plants is less clear (Nooden & Leopold, 1988). Some evidence suggests that ABA can trigger leaf senescence if large quantities of the hormone are applied (El-Antably *et al.* 1967; He & Jin 1999), while others have found that foliage ABA level increases as leaves age

especially as senescence peaks (Gepstein & Thimann 1980; He, Osaki, Takebe, Shinano & Wasaki 2005). In contrast, others have found that foliage ABA levels decrease during senescence (Uzelac *et al.* 2016), which suggests that ABA could be acting both synergistical or antagonistically with other drivers of leaf senescence depending on the timing of ABA increase, the specific tissue, and level of the hormone (Wingler, von Schaewen, Leegood, Lea & Paul Quick 1998; Pourtau *et al.* 2004; Rivero *et al.* 2007; Yang, Seo, Yoon & Park 2011; Kong *et al.* 2013; Uzelac *et al.* 2016; Asad *et al.* 2019).

An abundance of recent molecular work has provided compelling evidence for the importance of ABA biosynthesis and signaling pathways in regulating leaf senescence. Leaf senescence in response to ABA application is reduced in *Arabidopsis* and rice mutants of single or multiple ABA signaling genes, from receptors through to downstream kinases (Liang *et al.* 2014; Gao *et al.* 2016; Liu, Longhurst, Talavera-Rauh, Hokin & Barton 2016). Overexpression of some of these genes, particularly the ABA receptor *PYRABACTIN RESISTANCE1 LIKE 9* (*PYR9*), as well as downstream regulators of the ABA response, results in an enhanced leaf senescence phenotype in response to ABA application (Zhao *et al.* 2016; Mao *et al.* 2017). ABA is believed to enhance leaf senescence and cause early leaf necrosis in *Arabidopsis* through the regulation of a number of key transcription factors, mediated by reactive oxygen species (ROS) and calcium signals (Asad *et al.* 2019). When exogenous ABA is used to promote leaf senescence, in many studies across angiosperm species, significant changes to the expression of genes critical for ABA biosynthesis, signaling and senescence pathways are altered (Fan *et al.* 2015; Ren *et al.* 2018; Park *et al.* 2018; Asad *et al.* 2019). While this evidence links ABA with senescence, there are some studies which show that ABA application, or mutation to some ABA synthesis genes, conversely, delays leaf senescence in response to environmental stresses particularly osmotic stress, induced by salt or glucose (Wingler *et al.* 1998; Pourtau *et al.* 2004; Yang *et al.* 2011). Furthermore, the effect of ABA on leaf senescence appears to be highly dependent on ontogeny, with both young leaves, which rarely senesce and old, senescing leaves having similarly high levels of ABA (Powell 1975; Raschke & Zeevaart 1976; Weiler 1980; Cornish & Zeevaart 1984; Kane, Jordan, Jansen & McAdam 2020). It has also been suggested that the high ABA levels in senescing leaves may be due to a progressive decline in ABA sensitivity in the final stages of senescence, this insensitivity leading to more open stomata driving leaf desiccation in spite of high ABA levels in senescing leaves (Zhang, Xia, Zhang & Gan 2012; Zhang & Gan 2012). In an ABA deficient



sunflower mutant, senescence as leaves age occurs at the same rate as the wild type and foliage ABA levels similarly decline as leaves senesce (McAdam, Kane, Mercado Reyes, Cardoso & Brodribb 2022).

While molecular evidence provides support for a role of ABA in driving senescence, a potentially confounding aspect of this relationship comes from observations that closed stomata can induce senescence independently of ABA (Thimann and Satler, 1979*a,b*). These results suggest that the primary pathway by which ABA could promote, or enhance, the rate of leaf senescence might be via ABA driven stomatal closure, possibly leading to oxidative stress (Thimann & Satler 1979*b a*; Hensel, Grbić, Baumgarten & Bleecker 1993). In support of this idea, work on drought deciduous tree species indicates that leaf senescence is strongly associated with drops in leaf hydraulic conductance, decreasing leaf water potential ( $\Psi_L$ ) and stomatal closure, which continue to decline throughout senescence (Brodribb & Holbrook 2003). Low  $\Psi_L$  and reduced hydraulic conductance can also drive senescence in winter deciduous trees, with end-of-season senescence associated with declining  $\Psi_L$  and reduced hydraulic conductance due to accumulating tyloses in the xylem in *Castanea sativa* Mill. (Salleo, Nardini, Lo Gullo & Ghirardelli 2002). Declines in  $\Psi_L$  might implicate ABA, which is synthesized in leaves as cells lose turgor (Pierce & Raschke 1980; McAdam & Brodribb 2016), although this has not been measured..

Work in herbaceous models can be hard to apply to deciduous trees because the process of senescence in annual and perennial plants may be divergent (Lim *et al.* 2007). Many herbaceous and annual plants have linked flowering and seed-set with the onset of leaf senescence; the removal of flowers or fruit can substantially delay leaf senescence in many herbs (Leopold, Niedergang-Kamien & Janick 1959; Fan *et al.* 2020; Bucher & Römermann 2021). In contrast, woody plants tend to time senescence almost exclusively with either, or both, declines in temperature and shortening day length, independent of reproduction (Lang, Chen, Qian, Liu & Piao 2019). This further raises questions about whether ABA triggered leaf senescence in herbaceous model species translates to a mechanism that regulates the senescence of leaves in forests.

A recent study in four deciduous species found that foliage ABA levels increase during autumn, but only after senescence was initiated and chlorophyll *a* content had begun to decline (Zhang *et al.*, 2020). ABA has also been found to increase considerably after the onset of terminal embolism, including in three marcescent temperate species *Quercus falcata* Michx., *Betula*

*nigra* L., and *Carpinus betulus* L. after frost induced xylem embolism (McAdam et al., 2022). Experiments applying exogenous ABA to apple trees indicates a slight increase in senescence rates after the application of very high levels (3.78 mM, applied twice 7 days apart) of ABA as a foliage spray (Guak & Fuchigami 2001). Other studies have utilized the girdling of branches, or whole trees, to test whether phloem obstruction enhances leaf senescence (Dann, Wildes & Chalmers 1984; Lihavainen *et al.* 2021). ABA levels increase following girdling due to interruption to phloem ABA export and a subsequent accumulation in leaves (Dann *et al.* 1984; Lihavainen *et al.* 2021; López *et al.* 2015; Mitchell *et al.* 2017; Setter *et al.* 1980). Girdling experiments in peach and *Populus tremula* L. indicate that girdled tress, or branches, experience accelerated leaf senescence in autumn (Dann *et al.* 1984; Lihavainen *et al.* 2021). In these studies it is not clear whether ABA might be driving senescence indirectly by closing stomata, similar to the results of earlier work in leaf discs (Thimann & Satler, 1979*a,b*). Support of a stomatal pathway for the initiation of autumn senescence can be found in the accelerated rate of leaf senescence reported in *Populus tremuloides* Michx. in which leaves were herbivorized by epidermal leaf miners which fed exclusively on the lower epidermal cells of the hypstomatic leaves (Wagner et al., 2008). In plants with abaxial epidermal damage, including damage to guard cells, photosynthesis is greatly reduced because stomata cannot open, leaf lifespan is subsequently shortened by up to a month in these heavily herbivorized leaves (Wagner et al., 2008).

Here we sought to test whether: (1) increased endogenous ABA level drives autumn leaf senescence in four diverse winter deciduous trees, and (2) whether enhanced ABA levels driven by girdling triggers autumn leaf senescence. We chose a phylogenetically and ecologically diverse set of mature, field grown species including the gymnosperm *Ginkgo biloba* L. (Ginkgoaceae), angiosperm tree *Phellodendron amurense* Rupr. (Rutaceae), semi-frost tolerant shrub *Lonicera × purpusii* Rehder. (Caprifoliaceae) and marcescent angiosperm tree *Quercus falcata* (Fagaceae). We included a marcescent species because these species display a delayed leaf senescence and limited leaf abscission as adaptations to extend leaf lifespan and maximize late season photosynthetic gains (Abadía *et al.* 1996) or to deter winter herbivory (Svendsen 2001). We measured foliage ABA levels, leaf gas exchange,  $\Psi_L$ , and leaf chlorophyll *a* content in field grown during the autumn and also conducted a branch girdling experiment to determine the role of elevated levels of ABA on leaf senescence rates. *L. × purpusii* was included in the girdling experiment because leaves are highly tolerant of mild frosts with a very long leaf lifespan (Upson

& Kerley 2007), allowing us to determine senescence rates driven by increased ABA independent of temperature. We hypothesize that ABA levels increase during early senescence in winter deciduous species and that girdling enhances the rate of leaf senescence via higher ABA levels.

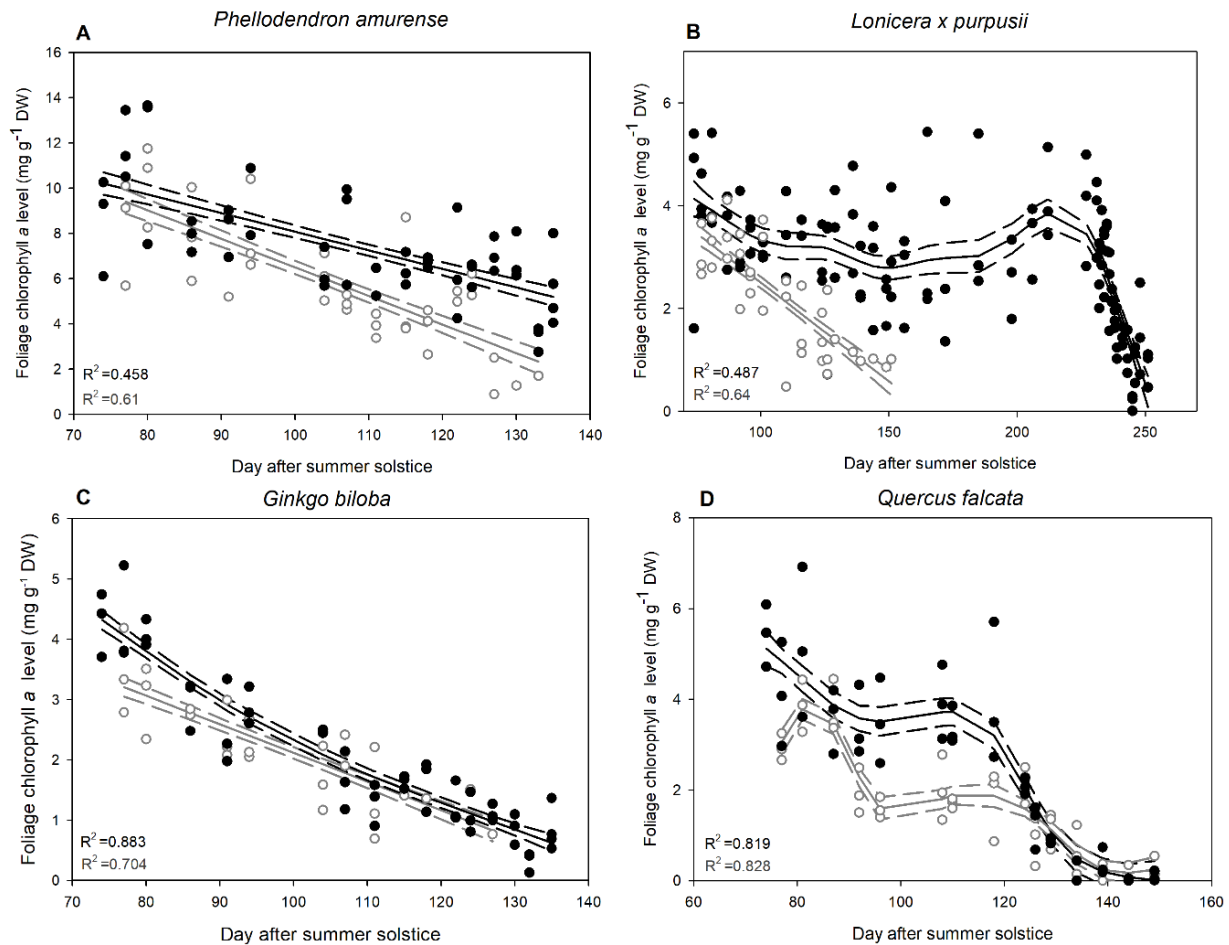


Figure 4-1. Foliage chlorophyll *a* content from mid-September 2020 in girdled (grey) and ungirdled (black) branches of *Phellodendron amurense* (A), *Lonicera × purpusii* (B), *Ginkgo biloba* (C) and *Quercus falcata* (D). The solid lines depict a general additive model with R<sup>2</sup> values shown, while the dashed lines demarcate standard error. Each point represents a single leaf.

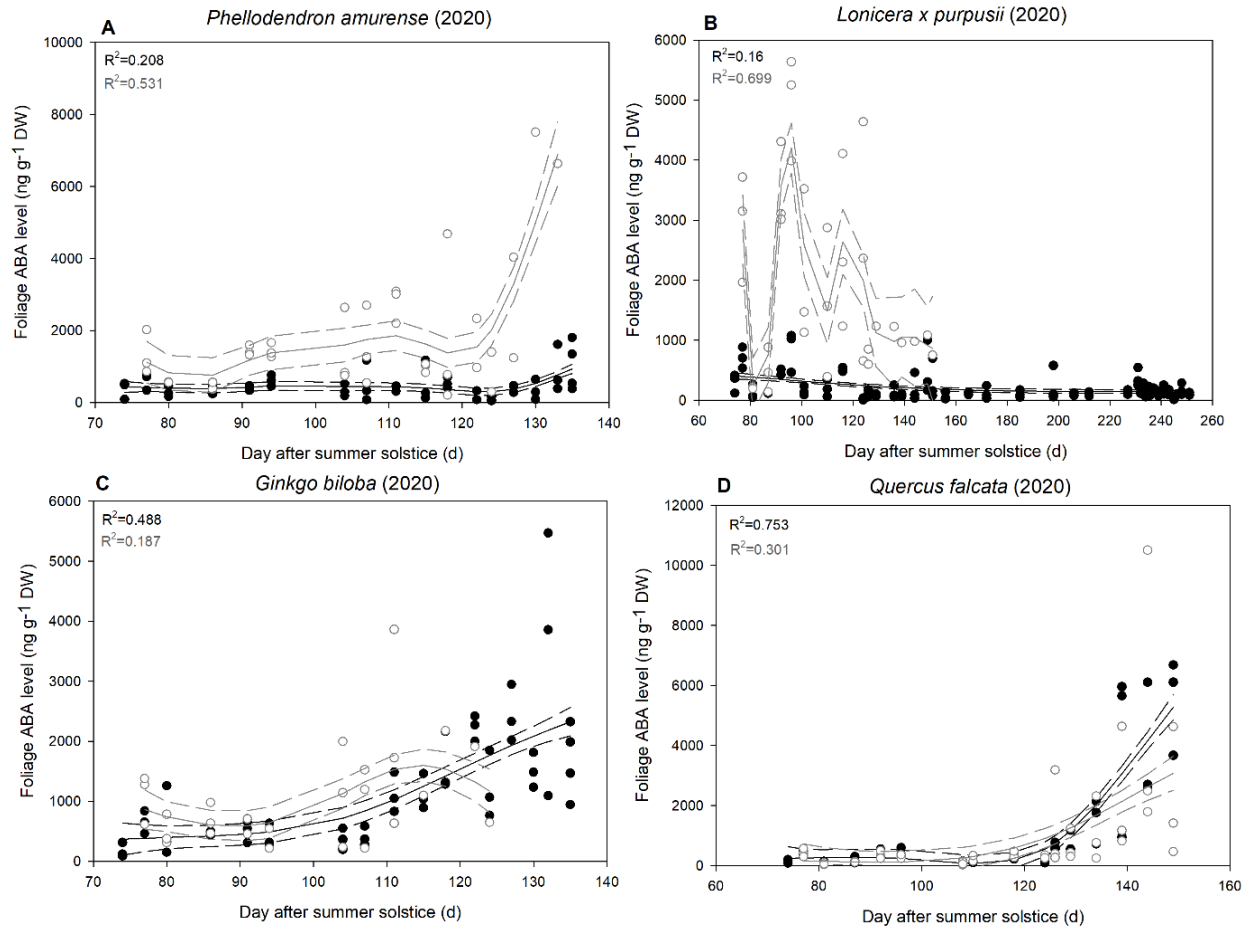


Figure 4-2. Foliage ABA content from mid-September 2020 in girdled (grey) and ungirdled (black) branches of *Phellodendron amurense* (A), *Lonicera × purpusii* (B), *Ginkgo biloba* (C) and *Quercus falcata* (D). The solid lines depict a general additive model with R<sup>2</sup> values shown, while the dashed lines demarcate standard error. Each point represents a single leaf.

## 4.3 Methods

### 4.3.1 Plant Material

The trees or shrubs of each species were established individuals growing on the grounds of the campus of Purdue University, West Lafayette, IN, USA, near Lilly Hall of Life Sciences (N 40.4233208, W 86.9167627). The measurements were made in early September until the middle of November when all leaves had abscised or were dead on the tree in the case of the marcescent *Q. falcata*.

### 4.3.2 Girdling experiment

74 days after the summer solstice (DAS) in 2020 three branches from two deciduous (*G. biloba* and *P. amurense*), one marcescent (*Q. falcata*), and one brevideciduous (*L. × purpusii*) species were girdled with a razor blade to remove the phloem while avoiding disruption of the xylem. After the phloem was removed, the xylem was covered in petroleum jelly and wrapped in cotton wool and tape to limit desiccation of the girdled xylem. The first measurement of the girdled branches was made three days after girdling. During each collection one leaf from each girdled branch and one leaf from a neighboring, intact, branch was selected and measured for gas exchange,  $\Psi_L$ , foliage ABA content, and foliage chlorophyll content. Measurements were made approximately twice a week on sunny days until all the leaves had fallen or died on the tree in the case of the marcescent *Q. falcata*.

### 4.3.3 Measurements

Measurements were made between 11:00 and 13:00. For each measurement three upper canopy leaves in full sun were selected. An infrared gas analyzer (LI-6800 Portable Photosynthesis System; LI-COR Biosciences, Lincoln, NE, USA) was set to ambient  $\text{CO}_2$ , ambient VPD and a saturating light intensity of  $1500 \mu\text{mol m}^{-2}\text{s}^{-1}$  to measure instantaneous  $\text{CO}_2$  assimilation and stomatal conductance. Immediately after recording assimilation and stomatal conductance the same leaf was detached from the tree and briskly wrapped in damp paper towel before being double bagged in Ziploc sandwich bags (SC Johnson, MI, USA) to prevent water loss for later determination of  $\Psi_L$  and sampling of tissue for pigment and hormone analysis. The bagged leaves were then placed in a dark bag to allow for  $\Psi_L$  equilibration for a minimum of 5 minutes.  $\Psi_L$  was measured using a Scholander pressure chamber (PMS Instrument Company, OR, USA) with microscope. After slow depressurization of the Scholander chamber, leaf tissue was harvested into two tubes to measure foliage ABA content and foliage chlorophyll content.

Foliage ABA was measured by physicochemical methods with an added internal standard. The mass of the fresh foliage sample was recorded ( $\pm 0.0001$  g, OHAUS Corporation, Parsippany, NJ, USA) and then the tissue was covered in  $-20^\circ\text{C}$  80% methanol in water ( $v v^{-1}$ ) containing  $250 \text{ mg l}^{-1}$  butylated hydroxytoluene (BHT), chopped into fine pieces and stored in a  $-20^\circ\text{C}$  freezer overnight. Methanolic extraction ensures unfettered and fettered ABA is extracted from the

chloroplasts (Georgopoulou & Milborrow 2012). The leaf tissue was homogenized and 15 ng of deuterium labeled [ $^2\text{H}_5$ ]ABA was added to each sample before extracting overnight at 4°C. An aliquot of supernatant, approximately 5 ml, was taken from each sample and dried down in a vacuum sample concentrator (Labconco, MO, USA), the ABA and internal standard was then resuspended in 200  $\mu\text{l}$  of 2 % acetic acid in water ( $v v^{-1}$ ), after which it was centrifuged at 14,800 RPM for 4 minutes. 100  $\mu\text{l}$  was taken for quantification of ABA and internal standard levels using an Agilent 6460 series triple quadrupole LC/MS (Agilent, CA, USA) according to (McAdam 2015). Following quantification, the homogenized leaf samples were dried at 70°C, and leaf dry mass was determined by subtracting the mass of the clean empty tube from the mass of the tube containing the dried homogenized leaf material.

Fresh mass determined was also determined for the second leaf sample which was used for foliage chlorophyll level quantification. After weighing the sample was covered in -20°C 100% acetone with 250  $\text{mg l}^{-1}$  BHT and roughly chopped and stored overnight at -20°C before being homogenized and extracted overnight at 4°C. A 100  $\mu\text{l}$  aliquot of supernatant was taken for chlorophyll *a* quantification using an Agilent 1100 high performance liquid chromatograph, with an Agilent 1100 G1315B diode array detector for UV-Visible spectrophotometric detection of pigments (Agilent, CA, USA). A Zorbax StableBond S8-C18 column (4.6 mm  $\times$  150 mm with 5- $\mu\text{m}$  particle size) (Agilent, CA, USA) and a quaternary pump (Agilent, CA, USA) according to McAdam et al. (2022). The amount of chlorophyll *a* was calculated in the injected sample volume using a standard curve of known quantities of chlorophyll *a*. Total extracted chlorophyll *a* was determined by then multiplying this number by the total volume of the acetone used to extract pigments. The total volume of acetone in the sample was determined gravimetrically by obtaining the mass of the total homogenized sample in acetone, then drying down that sample at 70°C and determining the mass of the dry sample. The volume of acetone was determined by multiplying the mass change and the density of acetone at standard atmospheric pressure at 180 m above sea level. Dry mass of the sample was then determined by cleaning the tube and redrying the vial to determine the mass of the clean tube which was subtracted from the mass of the dry sample with no acetone.

#### 4.3.4 Data analysis

Generalized additive models (GAM) (and standard errors) were fitted to chlorophyll *a* content and  $\Psi_L$  data over the season for each species. The timing of the onset of senescence was determined for each species and year as the day that recorded a 25% reduction of chlorophyll *a* content from the maximum according to the trend line of the GAM. The rate of senescence was taken as the number of days it took for foliage chlorophyll *a* content to decline from 75% of maximum to 50% of that maximum according to the GAM function. Single factor ANOVAs were used to determine if there was a significant difference in the timing of the onset of senescence and rate of senescence across species and years.

To determine the impact of girdling on foliar ABA levels the first 5 collections after girdling (~15 individual leaf samples) ending up to 22 days after girdling were pooled and averaged to estimate foliage ABA content after girdling. Significant differences between foliage ABA levels was determined using a Students T-test for each species.

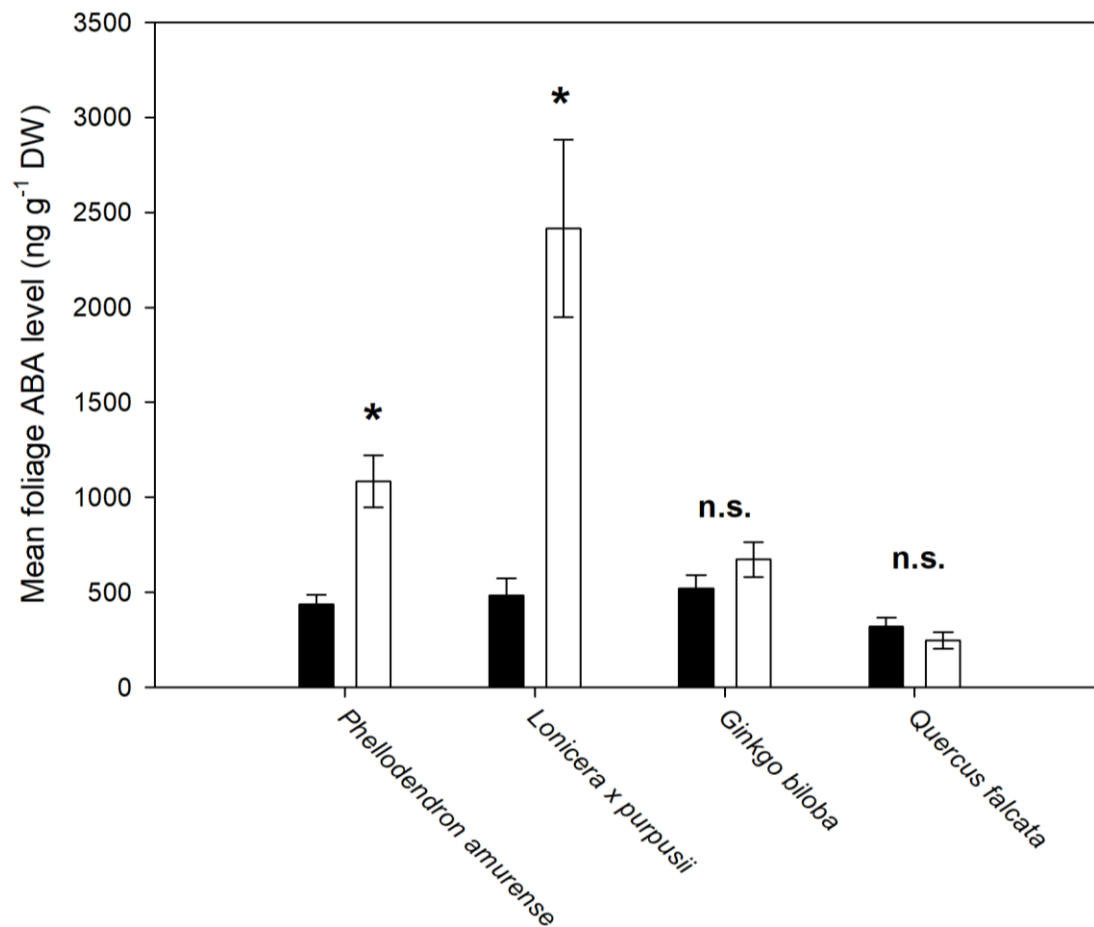


Figure 4-3. Mean and standard error of foliage abscisic acid levels in girdled (white) and ungirdled (black) branches from 3-20 days after girdling (n=15). \* denotes a significant difference in means ( $P < 0.05$ , T-Test).



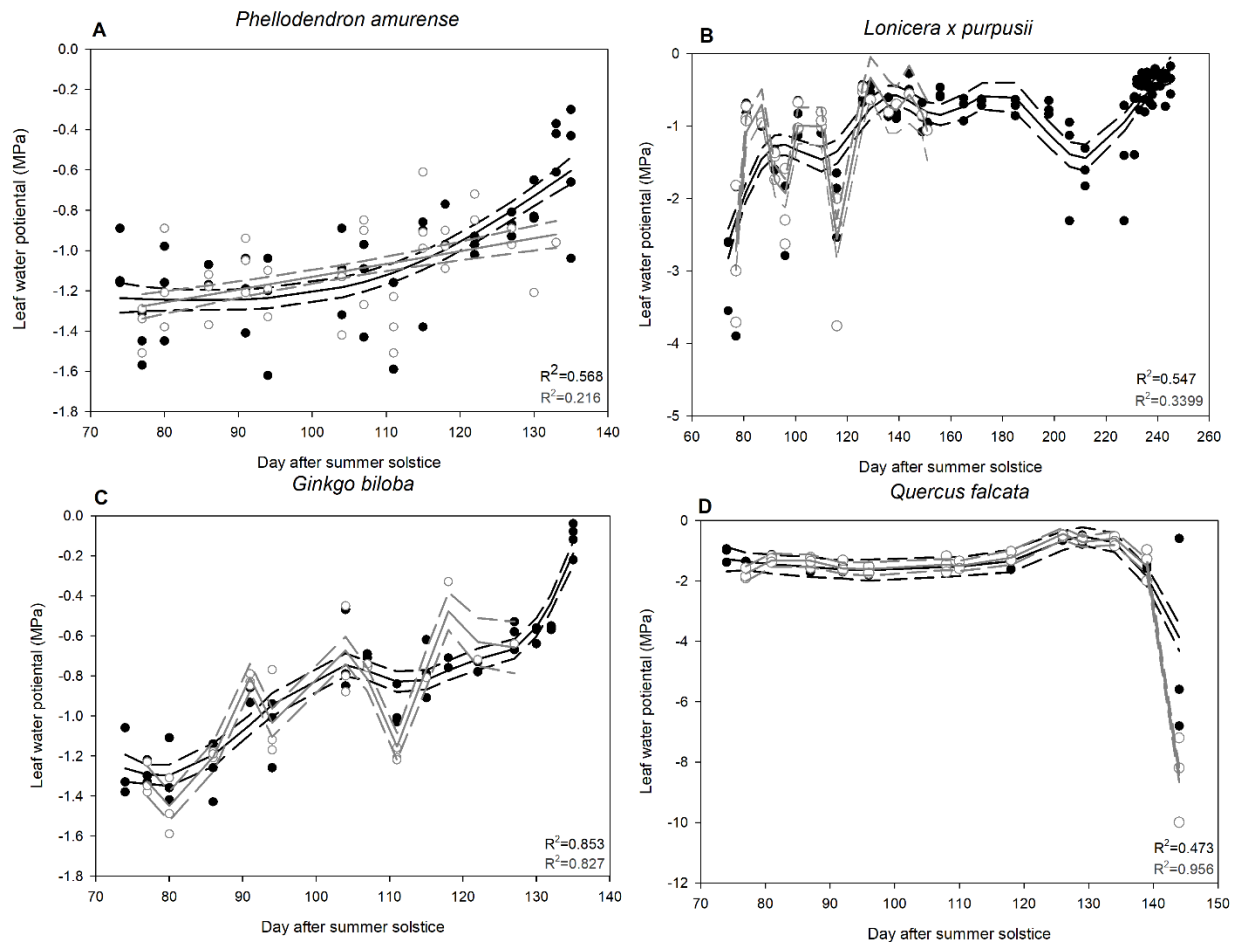


Figure 4-4. Leaf water potential from mid-September 2020 in girdled (grey) and intact (black) branches of *Phellodendron Amurense* (A), *Lonicera × purpusii* (B), *Ginkgo biloba* (C) and *Quercus falcata* (D). . The solid lines depict a general additive model (GAM) with  $R^2$  values shown, while the dashed lines demarcate standard error. Each point represents a single leaf.

## 4.4 Results

### 4.4.1 Variation in the onset and duration of senescence does not coincide with a change in ABA levels

In all species measured autumn triggered considerable declines in chlorophyll *a* content (Figure 4.1). The onset of chlorophyll *a* content decline varied depending on species and year sampled (Figure 4-1). In 2020 the onset of chlorophyll *a* content decline in intact branches occurred  $105 \pm 3$  days after the summer solstice (DAS) for *P. amurense*,  $131 \pm 25$  DAS for *L. × purpusii*, and  $87 \pm 9$  and  $\pm 2$  DAS for *Q. falcata* and *G. biloba*, respectively. The onset of senescence in the girdled branches fell within the standard error of the day of the onset of senescence of intact branches of *Q. falcata* ( $89 \pm 3$ ) and *G. biloba* ( $93 \pm 2$  DAS) but was different for the shrub *L. × purpusii* ( $98 \pm 4$  DAS) and *P. amurense* ( $95 \pm 2$  DAS). Average foliage ABA levels in intact branches in *P. amurense* declined from  $434 \text{ ng g}^{-1} \text{ DW} \pm 153 \text{ ng}$  at 74 DAS to  $296 \text{ ng g}^{-1} \text{ DW} \pm 99 \text{ ng}$  124 DAS after which it increased until leaf abscission (Figure 4-2). By the time foliar ABA levels began to increase chlorophyll *a* had already declined to 40% of initial levels. *Q. falcata* showed a similar trend with ABA content falling from  $243 \text{ ng g}^{-1} \text{ DW} \pm 378 \text{ ng}$  to  $176 \text{ ng g}^{-1} \text{ DW} \pm 262$  118 DAS after which it begins to increase at 118 DAS when chlorophyll *a* content had already declined to 37% of initial levels (Figure 4-2). In *L. × purpusii* we never observed an increase in foliage ABA content, the average declining by  $273 \text{ ng g}^{-1} \text{ DW}$  over the course of the season (177 days) (Figure 4.2). In *G. biloba* we observed a rapid increase in foliage ABA content 102 DAS when foliage chlorophyll *a* had already declined to 47% of initial levels (Figure 4-2).

### 4.4.2 Senescence onset timing and rate are enhanced by high ABA levels induced by girdling

*P. amurense* and *L. x purpusii* leaves on girdled branches senesced earlier than leaves on intact branches in the same plant (Figure 4-1). The onset of senescence in *P. amurense* was 10 days earlier in the girdled branches than in the intact branches. In *L. x purpusii* girdled branches senescence commenced 33 days earlier than the intact branches. The speed of senescence was also impacted by girdling with the decline from 75% to 50% of maximum chlorophyll *a* taking 30 days in intact *P. amurense* but only 18 days in the girdled branches. While in *L. × purpusii* leaves on the girdled branches senescence in 23 days unlike the intact branches which took 108 days to

decline from 75% to 50% of maximum chlorophyll *a* content. This earlier onset and accelerated senescence rate coincided with a much higher level of foliage ABA content in the leaves of the girdled branches of the *P. amurense* in the 20 days after girdling. In *P. amurense* the mean ABA level in the ungirdled branches was  $438 \pm 50 \text{ ng g}^{-1} \text{ DW}$  while the girdled branches maintained an average foliage ABA level of  $1084 \pm 136 \text{ ng g}^{-1} \text{ DW}$  during the same period after girdling (Figure 4-3). In *L. × purpusii* the average foliage ABA content in the 20 days after girdling increased in the girdled branches from  $483 \pm 92 \text{ ng g}^{-1} \text{ DW}$  in the ungirdled branches to  $2415 \pm 467 \text{ ng g}^{-1} \text{ DW}$  in the girdled branches.

This contrasts with the other two species *G. biloba* and *Q. falcata* which had similar levels of decline in chlorophyll *a* between girdled vs. ungirdled branches (Figure 4-1). *G. biloba* leaves started senescing around the same time in both girdled and intact branches at  $93 \pm 2$  and  $87 \pm 2$  DAS respectively. The time it took for *G. biloba* leaves to go from 75% to 50% of maximum chlorophyll *a* content was similar taking 15 days in intact branches and 18 days in girdled branches. This corresponded with no difference (One tail T-test:  $P= 0.0936$ ) in foliage ABA content after girdling from  $518 \pm 70 \text{ ng g}^{-1} \text{ DW}$  to  $673 \pm 91 \text{ ng g}^{-1} \text{ DW}$  in the girdled branches (Figure 4-3). In *Q. falcata* girdled branches began senescence  $89 \pm 3$  DAS while intact branches began  $87 \pm 9$  DAS. The rate of senescence was faster in girdled branches taking only 5 days to decline from 75% to 50% of maximum chlorophyll *a* while the intact branches took 34 days to decline the same percentage. The ABA content in the ungirdled leaves was not significantly different (One tail T-test:  $P= 0.1298$ ) than in the girdled leaves  $319 \pm 47 \text{ ng g}^{-1} \text{ DW}$  in the ungirdled branches while the girdled maintained an average foliage ABA level of  $247 \pm 43 \text{ ng g}^{-1} \text{ DW}$  in the 20 days after girdling (Figure 4-2).

#### 4.4.3 Senescence is not driven by a loss of hydraulic conductance

In 2020 *P. amurense* and *L. × purpusii* both displayed similar trends in  $\Psi_L$  between the girdled and intact branches during the autumn, with leaves becoming more hydrated through the season (Figure 4-4).  $\Psi_L$  77 DAS in leaves on girdled branches of *P. amurense* was around  $-1.38 \text{ MPa} \pm 0.06 \text{ MPa}$  at the same time the leaves on the intact branches were  $-1.44 \text{ MPa} \pm 0.08 \text{ MPa}$  (Two tailed T-test:  $P= 0.56$ ). Leaves of both intact and girdled branches became more hydrated to under  $-1 \text{ MPa}$  on the advent of abscission (Figure 4.4). In *L. × purpusii* at 77 DAS  $\Psi_L$  in girdled branches was  $-2.90 \text{ MPa} \pm 0.6 \text{ MPa}$  while the control branches were at a similar  $-2.84 \text{ MPa} \pm 0.55$

MPa (Two tailed T-test:  $P = 0.94$ ). After this day  $\Psi_L$  trended more hydrated until the leaves of the girdled branches had abscised all leaves around 151 DAS when  $\Psi_L$  of girdled branches reached  $-1.06 \text{ MPa} \pm 0.41$ , while control branches were at  $-0.81 \text{ MPa} \pm 0.14 \text{ MPa}$ .  $\Psi_L$  in intact branches continued to become more hydrated until all leaves were abscised around 245 DAS with  $\Psi_L$  near 0 MPa (Figure 4-4). This increase in  $\Psi_L$  through the fall can be seen across all years in *P. amurense* and *G. biloba* (Figure 4-4). The only species in which  $\Psi_L$  did not increase was in the marcescent *Q. falcata* in which  $\Psi_L$  declined dramatically after 134 DAS in 2020, when chlorophyll *a* content had declined more than 90% of maximum (Figure 4-4).

*Senescence reduces assimilation.*

CO<sub>2</sub> assimilation forms a strong relationship with stomatal conductance across all species and years measured, with an  $R^2$  of 0.8649, P-value of less than 0.0001, F-statistic 2490.07, and DF = 390 (Figure 4-5). This relationship rises to a maximum at  $19.4316 \mu\text{mol m}^{-2} \text{ s}^{-1}$  of CO<sub>2</sub> with a water loss of  $0.5134 \text{ mol m}^{-2} \text{ s}^{-1}$  of H<sub>2</sub>O (Figure 4-5). CO<sub>2</sub> assimilation forms a slightly weaker but still significant linear relationship with chlorophyll *a* content (Figure 4-6). CO<sub>2</sub> assimilation forms a relationship with an  $R^2$  of 0.3595 and a P-value of less than 0.0001, F-statistic of 199.84, and DF = 357 (Figure 4-6). As chlorophyll *a* content declines so does CO<sub>2</sub> assimilation (Figure 4-6).

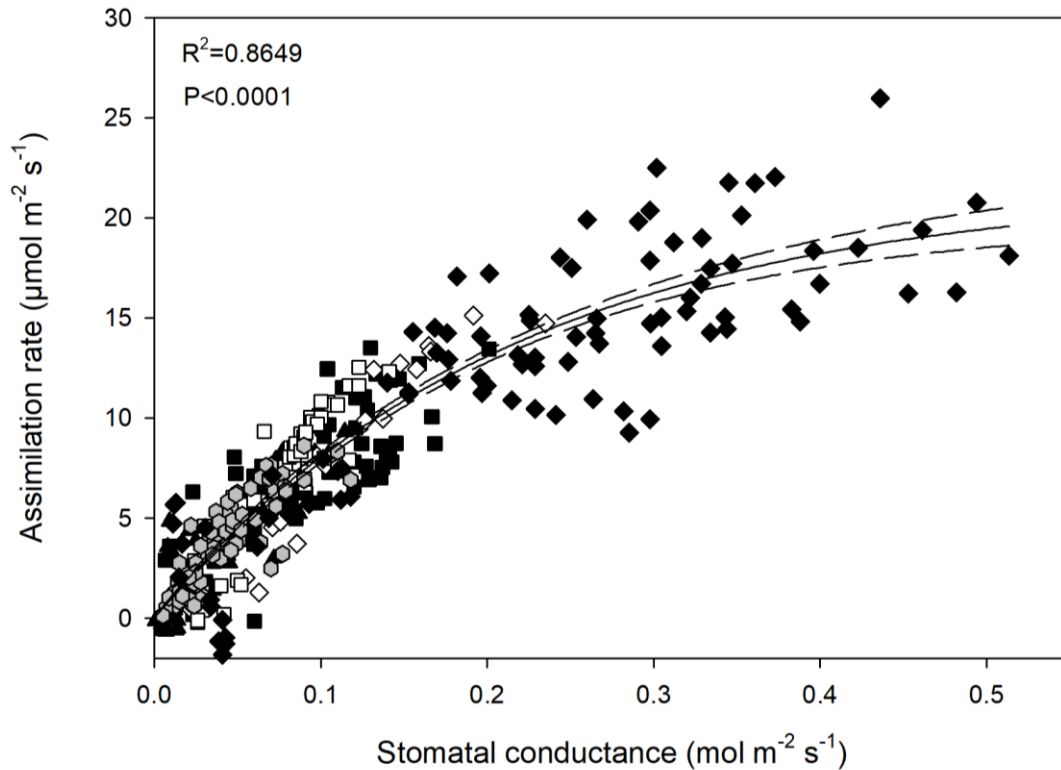


Figure 4-5. The relationship between CO<sub>2</sub> assimilation rate and stomatal conductance measured in leaves from all species from late summer until the end of the autumn. The solid line fits an Exponential Rise to Maximum, Single, 2 Parameter (Assimilation rate= $21.519 \cdot (1 - (-4.6816 \cdot \text{stomatal conductance}))$ ). The dashed lines represent the 95% confidence interval of the fit curve. Each point represents one leaf measured. Squares represent *Phellodendron amurense*, hexagons *Lonicera x purpusii*, triangles *Ginkgo biloba* and diamonds *Quercus falcata*. and. Black symbols indicate data collected in 2018, white 2019, and grey in 2020.

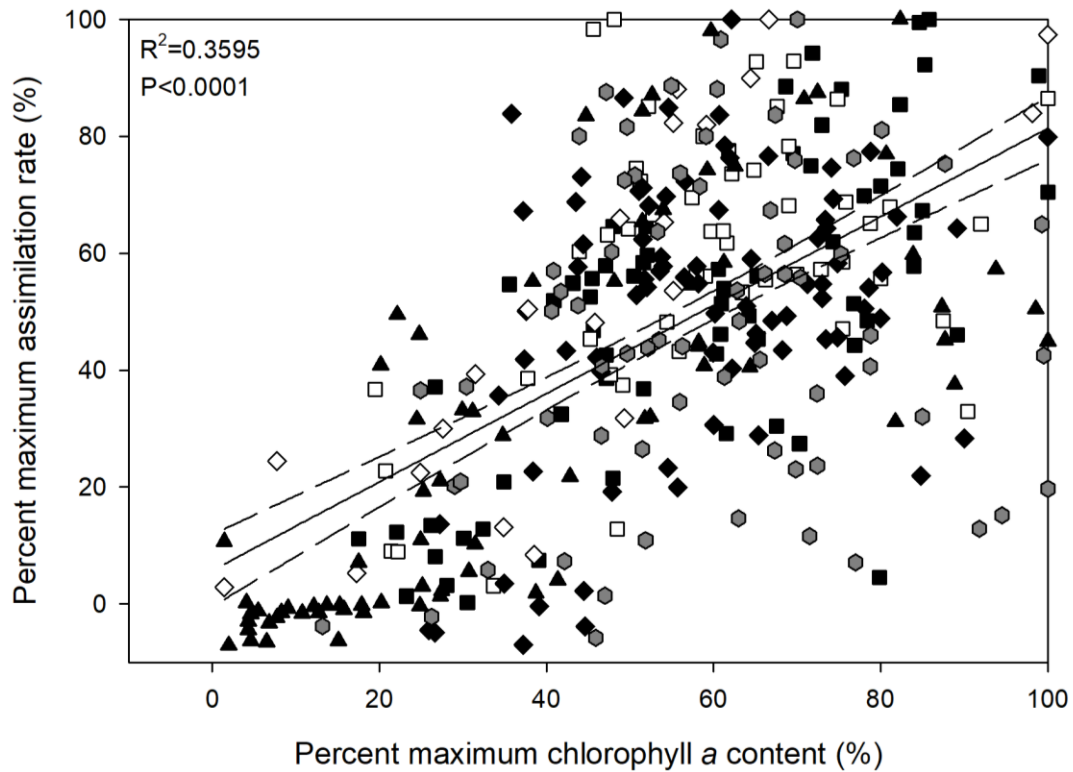


Figure 4-6. The relationship between the percentage of maximum chlorophyll *a* content in leaves and percentage of maximum CO<sub>2</sub> assimilation rate in all species from late summer until the end of the autumn. The solid line fits an linear relationship, (Percent maximum assimilation rate= $5.7428+0.7559 \times$  Percent maximum chlorophyll *a* content). The dashed lines represent the 95% confidence interval of the fit curve. Each point represents one leaf measured. Squares represent *Phellodendron amurense*, hexagons *Lonicera x purpusii*, triangles *Ginkgo biloba* and diamonds *Quercus falcata*. Black symbols indicate data collected in 2018, white 2019, and grey in 2020.

## 4.5 Discussion

### 4.5.1 ABA can enhance deciduous leaf senescence but does not increase at the onset of senescence

Contrary to some observations in *Zea mays* L. and *Avena sativa* L. (Gepstein & Thimann 1980; He *et al.* 2005) we did not observe an increase in foliage ABA content at the onset, or for most of the duration of leaf senescence, in winter deciduous species. In our girdling experiments we did observe that increased foliar ABA content during the autumn can trigger an earlier onset and faster senescence rate of two winter deciduous species (*P. amurense* and *L. × purpusii*). Our data are similar to that of four deciduous species measured in China, in which ABA levels were found to increase in deciduous tree leaves but only after chlorophyll *a* content had begun to decline (Zhang *et al.* 2020). Our data also concurs with many studies in *Arabidopsis* and other herbaceous species that ABA either exogenously applied, soaking leaf segments in ABA solutions, and mutation to the ABA synthesis or signaling pathway can enhance and trigger leaf senescence (El-Antably *et al.* 1967; Chin & Beevers 1970; Gepstein & Thimann 1980; Philosoph-Hadas *et al.* 1993; Fan *et al.* 1997; Hung & Kao 2003; Liang *et al.* 2014; Gao *et al.* 2016; Liu *et al.* 2016; Zakari *et al.* 2020).

The absence of an increase in ABA levels prior to the onset of senescence, or during senescence, in intact branches implies that foliage ABA is not necessary for triggering the onset of autumn leaf senescence in winter deciduous trees. We did observe an increase in foliage ABA content just before abscission or leaf death by heavy frost in the marcescent species *Q. falcata*. This could be related to phloem disruption during the formation of the abscission zone or frost induced embolism causing a death-associated spike in foliage ABA content, as has been reported in marcescent species, including *Q. falcata* (McAdam *et al.*, 2022). Our observations that ABA does not increase at the onset of leaf senescence should trigger further investigation into the nature of ABA dynamics in *Arabidopsis* leaves, and other herbaceous species during natural aging, to determine if foliage ABA levels increase at the onset and during leaf senescence. We also do not know if ABA is likely to trigger leaf senescence if a plant is experiencing stressful conditions that normally trigger increases in foliar ABA, such as drought.

Even though we did not observe increases in foliage ABA levels during the onset of leaf senescence in any of our observed species over the three years of data collection, our experimental

data indicates that high levels of ABA may be able to enhance leaf senescence in deciduous species. The two species that showed significantly higher levels of foliage ABA after girdling (*P. amurense* and *L. × purpusii*) showed a corresponding earlier onset of senescence and in *P. amurense* a more rapid decline in chlorophyll *a* content through the autumn. This was not observed in *G. biloba* in which foliage ABA content did not increase significantly following girdling. In the marcescent *Q. falcata* no change in foliage ABA levels was also observed after girdling but we did observe a slightly enhanced degradation of chlorophyll *a* following girdling, with the girdled branches appearing have an early decline in chlorophyll *a* before matching girdled branches later in the season. This could be evidence of an additional factor driving girdling-induced changes in chlorophyll degradation, such as changes in source sink feedback triggering faster senescence as suggested by Zani et al. (2020). Our results confirm earlier work in herbaceous species which indicates that ABA can augment leaf senescence (Guak & Fuchigami 2001; Zhao et al. 2016; Mao et al. 2017; Asad et al. 2019) and other studies that have seen increases in leaf senescence activity after girdling (Dann et al. 1984; Lihavainen et al. 2021).

#### 4.5.2 Leaf dehydration and loss of conductance does not drive annual leaf senescence

In our deciduous species we did not observe declining  $\Psi_L$ . In *Castanea sativa* Salleo et al. (2002) suggested that the gradual accumulation of occlusions in the xylem lead to decreases in hydraulic conductance, which were the primary driver for leaf senescence in deciduous species. We did not see any evidence of hydraulic failure driving senescence in our species, with  $\Psi_L$  becoming more hydrated as stomata closed and leaves senesced. We could hypothesize that potential declines in hydraulic conductance in *C. sativa* would coincide with higher levels of ABA, which one might expect as ABA levels are closely linked with leaf water status (Pierce & Raschke 1980; McAdam & Brodribb 2016), and that this potential accumulated ABA drove leaf senescence observed by Salleo et al. (2002). Declining predawn  $\Psi_L$  during the autumn have been observed in two cultivars of *Prunus dulcis* Batsch. grown in southeastern Spain, this decline in predawn  $\Psi_L$  did not translate to a decline in midday  $\Psi_L$  which remained around -2 MPa the whole season, similar results were observed in pot grown seedlings of *Magnolia grandiflora* L. and *Liquidambar styraciflua* L. (Augé & Stodola 1989; Ruíz-Sánchez, Sánchez-Blanco, Planes, Alarcón & Torrecillas 1993). In contrast in six evergreen conifer species native to Wyoming both morning



and midday stem water potentials increases towards 0 MPa during autumn, similar to what we observed here (Smith, Young, Carter, Hadley & McNaughton 1984).

#### **4.5.3 Stomata close during senescence and may drive autumnal leaf senescence**

While ABA levels may play a role in accelerating the onset and in some cases the rate of leaf senescence in deciduous species the interaction between ABA and stomatal closure on triggering or accelerating senescence cannot be ruled out. Thimann & Satler (1979*a,b*) found that stomatal aperture played a critical role in leaf senescence activation and speed, independent of ABA. Further work is required to elucidate if ABA is directly activating and accelerating leaf senescence in winter deciduous trees, or if ABA acts indirectly via reductions of stomatal conductance and photosynthesis to drive leaf senescence. Leaf and whole plant non structural carbohydrate status may play a role in triggering senescence as has been suggested by Zani *et al.*, 2020 and Westgeest *et al.*, (2022). Westgeest *et al.* (2022) found that stomatal conductance can be driven by leaf starch status. We found the relationship between stomatal conductance and CO<sub>2</sub> assimilation stays consistent (Wong, Cowan & Farquhar 1979) and at no point during the leaf senescence process does CO<sub>2</sub> assimilation and stomatal conductance decouple as might have been seen if stomata opening during the senescence process due to ABA insensitivity occurred as suggested by Zhang *et al.* (2012) and Zhang and Gan (2012) based on *Arabidopsis*. Our observed seasonal decline in stomatal conductance matches data found in drought deciduous forests and evergreen conifers in temperate climates (Smith *et al.* 1984; Brodribb & Holbrook 2003).

#### **4.5.4 Conclusion**

We find that ABA enhances leaf senescence in some winter deciduous species, but in unstressed plants ABA levels do not increase until the very end of leaf life after chlorophyll levels have almost completely declined. We observed that in deciduous trees as temperatures drop and days shorten, chlorophyll *a* begins to decline at the same time stomatal conductance and photosynthesis declines, while  $\Psi_L$  becomes less negative as transpiration declines.

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## CHAPTER 5. SPATIAL AND TEMPORAL FREEZING DYNAMICS OF LEAVES REVEALED BY TIME-LAPSE IMAGING

The text and results of this chapter are under review at *New Phytologist*:

### 5.1 Abstract

- Freezing temperatures mark the end of leaf life for many species at high latitudes, yet the leaves of some species can survive these events. Survival could be due to lower freezing points in leaf tissues or an ability to recover metabolic function after freezing.
- We deployed continuous imaging, fine wire thermocouples and leaf gas exchange to the leaves of the brevideciduous Purpus honeysuckle (*Lonicera × purpusii*) a species which has leaves that can tolerate temperatures below 0°C, to investigate in-situ freezing points and the capacity for tissue recovery from freezing.
- We found that while ex-situ xylem sap freezes near 0°C, in-situ xylem sap and leaf mesophyll have freezing points below -4°C in the field. Ice formation in leaves exposed to natural frost initiates in the mesophyll before spreading to veins. Leaf photosynthetic rate measured in leaves that have been exposed to a rapid freeze do not recover on thawing, but do when exposed to a slow, natural freezing to -10°C.
- We present a new method time-lapse image-based method for observing the spatial formation and timing of freezing events in leaves, and suggest that in situ and ex situ freezing points for xylem sap can differ by more than 4°C.

### 5.2 Introduction

Freezing air temperatures mark the end of woody-plant primary productivity at high latitudes (Baldocchi *et al.* 2005). To survive this seasonal extreme, deciduous species shed senesced leaves and enter winter dormancy (Clements & Ludlow 1977; Sakai & Larcher 1987; Bassow & Bazzaz 1998; Lubbe & Henry 2019), while the leaves of evergreen and brevideciduous species tolerate repeated freezing and thawing cycles through all, or most of, the winter (Taneda & Tateno 2005; Koehler, Center & Cavender-Bares 2012). The ability of plants to retain leaves

that can survive multiple freeze-thaw cycles ensures carbon assimilation during brief warm periods through winter and in early spring (Chabot & Hicks 1982; Sprugel 1989; Miyazawa & Kikuzawa 2005; Hughes, Smith, Hughes & Smith 2007). Consequently, leaf freezing tolerance is adaptively relevant and can determine plant community composition at high latitudes (Tranquillini 1982; Inouye 2000; Walker *et al.* 2004; Stuart, Choat, Martin, Holbrook & Ball 2007; Löffler 2007). As average temperatures trend warmer, a shift towards earlier bud burst in temperate deciduous forests may increase the exposure of sensitive species to spring frosts normally avoided due to longer bud dormancy (Menzel, Helm & Zang 2015; Lamichhane 2021). The economic consequences for horticultural trees of these changes are considerable, where leaves and flowers are at a huge risk from sudden frosts (Rodrigo 2000; Zohner *et al.* 2020). Despite the risks of freezing to plants, studies investigating the mechanism of freezing survival in plants *in-situ* have declined significantly in the last 20 years (Wisniewski, Gusta, Fuller & Karlson 2009), such that we still do not have a good understanding of the spatial pattern of freezing in leaves, or whether the nature of freezing determines the ability of leaves to recover photosynthetic function on thawing.

Leaves that can survive multiple freezing events do so by one of two major strategies, by supercooling organs to avoid ice nucleation (Sakai & Larcher 1987), or tolerance of multiple freeze events (Burke, Gusta, Quamme, Weiser & Li 1976). The supercooling response provides transient protection for plants experiencing infrequent freezing events (Thomas & Barber 1974; Sakai & Larcher 1987; Rada, Goldstein, Azocar & Torres 1987; Squeo, Rada, Azocar & Goldstein 1991; Larcher, Meindl, Ralser & Ishikawa 1991; Ball *et al.* 2002). This strategy is widely adopted by plants that experience infrequent freezing events, but fails if the plant experiences temperatures beyond the supercooling threshold limit (Sakai & Larcher 1987; Squeo *et al.* 1991; Arias, Bucci, Scholz & Goldstein 2015). The other strategy employed by plants to survive freezing temperatures is being able to tolerate or recover from multiple freezing events (Sucoff 1969; Sakai & Larcher 1987; Sperry & Sullivan 1992; Davis, Sperry & Hacke 1999; Cochard, Lemoine, Améglio & Granier 2001; Feild & Brodribb 2001). This involves cell survival from complete freezing of water in the apoplast. When leaf freezing occurs, apoplastic freezing generates a brief temperature spike referred to as the first exotherm, followed by a second isotherm when the cell sap freezes, once the second isotherm occurs it is assumed cell function is irrecoverable (Stergios & Howell 1973; Sakai & Larcher 1987; Squeo *et al.* 1991; Ashworth 1993; Taschler & Neuner 2004). It is

speculated that some plants will osmotically adjust cells during the autumn and winter to reduce the temperature threshold of symplastic freezing (Gail 1926; Levitt 1957; O'Neill 1983). The primary causes of freezing damage to cells in woody plants is by ice nucleation piercing cell membranes (Guy 1990), extracellular ice formation causing cell dehydration (Gusta, Burke & Kapoor 1975), or freezing and thawing in the xylem tissue inducing embolism that breaks the water transport stream, leading to dehydration and death of upstream tissues (Sperry, Nichols, Sullivan & Eastlack 1994; Langan, Ewers & Davis 1997; Utsumi, Sano, Funada, Fujikawa & Ohtani 1999; Mayr, Gruber & Bauer 2003; Koehler *et al.* 2012).

The most popular method to measure plant freezing temperatures, the timing of freezing, and freezing progression across organs is infrared video thermography where temperature is measured using the output of thermal energy in the form of infrared radiation (Fuller & Wisniewski 1998; Stier, Filiault, Wisniewski & Palta 2003). This technique allows for relatively easy analysis of plant tissue freezing in many types of plant tissue, but does require relatively costly, specialized cameras (Fuller & Wisniewski 1998; Morales, Sierra-Almeida & Kalin Arroyo 2023). Another way that *in situ* freezing has been monitored in the field is via nuclear magnetic resonance microscopy which uses magnetic resonance to observe state changes in, predominantly, excised plant tissues as liquid water becomes ice (Hills & Remigereau 1997; Ide, Price, Arata & Ishikawa 1998). This technique has a very high spatial tissue-scale resolution, but it is expensive and challenging to deploy in the field (Hills & Remigereau 1997; Ide *et al.* 1998; Ishikawa *et al.* 2009).

In this study we utilized the brevidiciduous Purpus honeysuckle (*Lonicera × purpusii* Rehder. (Caprifoliaceae)) which is an F1 hybrid between *L. fragrantissima* Lindl. & Paxton and *L. standishii* Jacques (Dulić 2012; USDA 2022) to examine the freezing point of leaf tissues and *ex situ* xylem sap. The genus *Lonicera* contains multiple species of shrub, liana and creeper that are highly invasive, particularly in eastern North America (Miller & Gorchoff 2004; Schierenbeck 2004; Love & Anderson 2009), as well as freezing tolerant (McEwan, Birchfield, Schoergendorfer & Arthur 2009; Brailko & Gubanova 2014; Tofig, Shalala & Aisel 2022). One of the primary causes of Eastern Asian *Lonicera* spp. naturalization in eastern North America is the ability of leaves of these species to tolerate mild freezing conditions in both spring and fall which allows for a prolonged growing season when compared to native shrubs, increasing annual assimilation while overstory trees are leafless (McEwan *et al.* 2009; Fridley 2012; Smith 2013). To investigate the nature of freezing in *L. × purpusii* we developed a novel time-lapse imaging method, using a

RaspberryPi driven camera and manifold similar to that which was originally developed to detect embolism in leaves and stems (Brodribb *et al.* 2016), to observe the progression and timing of natural winter freezing and thawing events in the field in leaves of *L. × purpusii*. Our new method analyzes pixel brightness to map the spatial patterns of freezing and thawing in leaf tissue and can be used in the field to track freezing in tissues exposed to natural winter frost events. In addition to mapping freezing and thawing events in leaves, we also test the effect of freezing speed on leaf photosynthetic recovery and damage in this species.

## 5.3 Methods

### 5.3.1 Plant material

A ten-year-old specimen of *L. × purpusii* was used for these experiments, grown outside on the campus grounds of Purdue University, West Lafayette, Indiana, 47907, USA (40.422833 N, -86.916837 W) on the South facing side of a building. Measurements were taken between January and March 2021. For experiments conducted in the lab on excised branches, stems longer than the longest vessel was always used to avoid inducing embolism. The mean length of the longest vessel, determined by air-injection, was  $34.5 \pm 1.96$  mm (n=4).

### 5.3.2 Visualizing leaf freezing

To visualize the freezing of leaves *in-situ* Raspberry Pi 4 Model B (Raspberry Pi, United Kingdom) clamps were used (Brodribb *et al.* 2016). To capture freezing events *in-situ* the Raspberry Pi clamp was attached to an unfrozen, green leaf still attached to the shrub on the afternoon of a day when temperatures were above freezing, but before a night when minimum temperatures were forecast to drop below -20°C, and also forecast to rise above freezing the following day (the night of the 28<sup>th</sup> January 2021, DOY 28 – DOY 29). Leaves were imaged every 3 min. Leaf temperature was monitored using a fine wire thermocouple placed on the leaf inside the clamp and attached to the CR850 data logger. To analyze freezing dynamics images were assembled into an image stack using Fiji image analysis software (U. S. National Institutes of Health, Maryland, USA).

The image stack was divided into a 450 x 450-pixels section which included midrib, minor veins, and mesophyll (FOV 25mm<sup>2</sup>). This stack was then divided into 100 further divisions (45 x

45-pixels, or a FOV of 0.25 mm<sup>2</sup>) and mean pixel brightness (mean RGB value for the whole section) was extracted from each slice of all 100 subsections. The onset of freezing was determined as a 10% increase in mean initial pixel brightness, and onset of thawing was determined as a 10% decrease in mean maximum pixel brightness. In order to test the spatial limits of this method a 90 x 90-pixel section (FOV 1 mm<sup>2</sup>) was analyzed in the same way with each sub-section being 9 x 9-pixels (FOV 0.01 mm<sup>2</sup>) focusing on an areole. The raw leaf temperature data during freezing and thawing was fitted with a linear regression to determine the temperature at which each subsection froze or thawed.

### **5.3.3 Determination of the freezing point of leaf, stem, and xylem sap**

To determine freezing points fine-wire thermocouples attached to leaves and connected to a CR850 data logger (Campbell Scientific, Utah, USA) were used to measure temperature, logged every 1s. Thermocouples were folded so they remained in constant contact with the leaf surface. To measure intact xylem freezing points, bark and cambium were carefully removed by hand to avoid causing embolism in the underlying tissues and the exposed area was washed with deionized water to eliminate cellular contents from the phloem and cambium. Thermocouple wires were then tied around the stems with the tip of the thermocouple placed against the exposed xylem tissues. *Ex-situ* xylem sap was collected from branches with at least 10 leaves. The cambium and phloem tissues were removed from the cut end to reveal the xylem, and the stems were then placed into a Scholander Pressure Chamber (PMS Instrument Company, Oregon, USA) which was laid on its side and gently pressurized using N<sub>2</sub> gas until 0.05 MPa beyond the endpoint when xylem sap began to flow from the cut end (approximately 0.3 MPa of pressure). The sap, approximately 1 ml, was collected in 2 ml tubes over 10 mins.

To determine freezing points all tissues were exposed to the same treatment. Samples were placed into a large plastic zip-lock bag that contained damp paper towel to reduce evaporation. This bag was then placed into a Styrofoam box to provide a uniform air temperature, and to slow freezing times, which was placed into a -20°C freezer (Roper Technologies, Florida, USA). The time to freezing took approximately 60 mins, with an average cooling speed of -1°C min<sup>-1</sup>. Temperatures were recorded until after the freezing exotherm. After observing the exotherm the samples were left in the freezer to ensure no additional freezing events occurred. The freezing point was determined to be the highest temperature recorded immediately after the freezing

exotherm (Beck, Schulze, Senser & Scheibe 1984; Woo & Mujumdar 2010). Three samples of each tissue type or xylem sap were used to determine the mean freezing point.

#### **5.3.4 The effect of freezing speed on recovery**

To test the effect of how fast the decline in temperature to freezing effects photosynthetic recovery in *L. × purpusii* branches longer than the longest vessel was cut under deionized water and leaves placed in plastic bags containing damp paper towel. Two high power Schott KL 2500 LCD lights (Schott AG, Germany) were used to irradiate the branches for 1 h. After the hour of high light gas exchange in 2 leaves from 3 branches was measured using a LI-6800 Portable Photosynthesis System (LI-COR Biosciences, NE, USA) set to a vapor pressure deficit of 1.2 kPa, CO<sub>2</sub> of 400 ppm, and light intensity of 1500 PAR. Fine wire thermocouples were then attached to leaves with tape so temperatures could be monitored via the CR850 data logger (Campbell Scientific, Utah, USA). Branches were then re-bagged with damp paper towel and either placed directly into a -20°C freezer in which leaves cooled rapidly (-10°C min<sup>-1</sup>) or placed inside a Styrofoam box, to slow cooling, and then placed into a -20°C freezer. Leaf temperatures were monitored as they declined and the freezing exotherm was noted. We allowed temperature to fall after the exotherm to around -10°C. Branches were then removed from the freezer and allowed to rise to room temperature after which they were recut under water and put back under high light for 1 h and photosynthesis was remeasured in the same leaves using the LI-6800 using the same environmental settings.

Leaf water potential was measured in neighboring leaves on the same branch immediately after measuring photosynthesis before and after freezing by wrapping leaves in damp paper towel and placing them in a sealed plastic bag and allowing to equilibrate in a dark drawer for 10 min before measurements were made in a Scholander Pressure Chamber (PMS Instrument Company, Oregon, USA).

#### **5.3.5 Assessing maximum photosynthetic rates of leaves exposed to air and buried under snow under repeated freezing and thawing.**

To test whether damage occurred to leaves by freezing or senescence, and the lowest temperature from which *L. × purpusii* leaves can survive after freezing, maximum photosynthetic rates were measured around 4 times per week starting on the DOY 28, 2021 which preceded a

winter snow storm on the DOY 30 2021 (during which 150 mm of snow fell over 48 h), and until snow melt on the DOY 54, 2021. After the snow fall lower branches with leaves buried by the snow were marked with tape for measurement of leaves upon snow melt. To measure maximum assimilation rate branches longer than the longest vessel were cut under deionized water, bagged with a zip top bag containing damp paper towels and transported to the lab. Once inside the branches were placed under lights for 1 h then gas exchange in three leaves was measured in a LI-6800 set to a vapor pressure deficit of 1.2 kPa, CO<sub>2</sub> of 400 ppm, and light intensity of 1500 PAR and temperature of 22°C. All branches measured before the snow storm were exposed to the air for the duration of the measuring window. After the snow storm the upper branches that were exposed to the air were compared to leaves on lower limbs that were buried under snow since the snow fall event. Minimum nighttime air temperatures were recorded at the Purdue University Airport located 2 km from the study plant.

### **5.3.6 Data analyses and statistics**

The freezing temperatures of leaf, xylem, and *ex-situ* xylem sap and the recovery of leaf assimilation and water potential were analyzed using a one-way ANOVA with a Tukey's HSD post hoc test. The effect freezing temperature of vein vs mesophyll in the 90x90 section were compared using two-way t-tests by subtracting the initial from final leaf water potential, assimilation, and stomatal conductance.

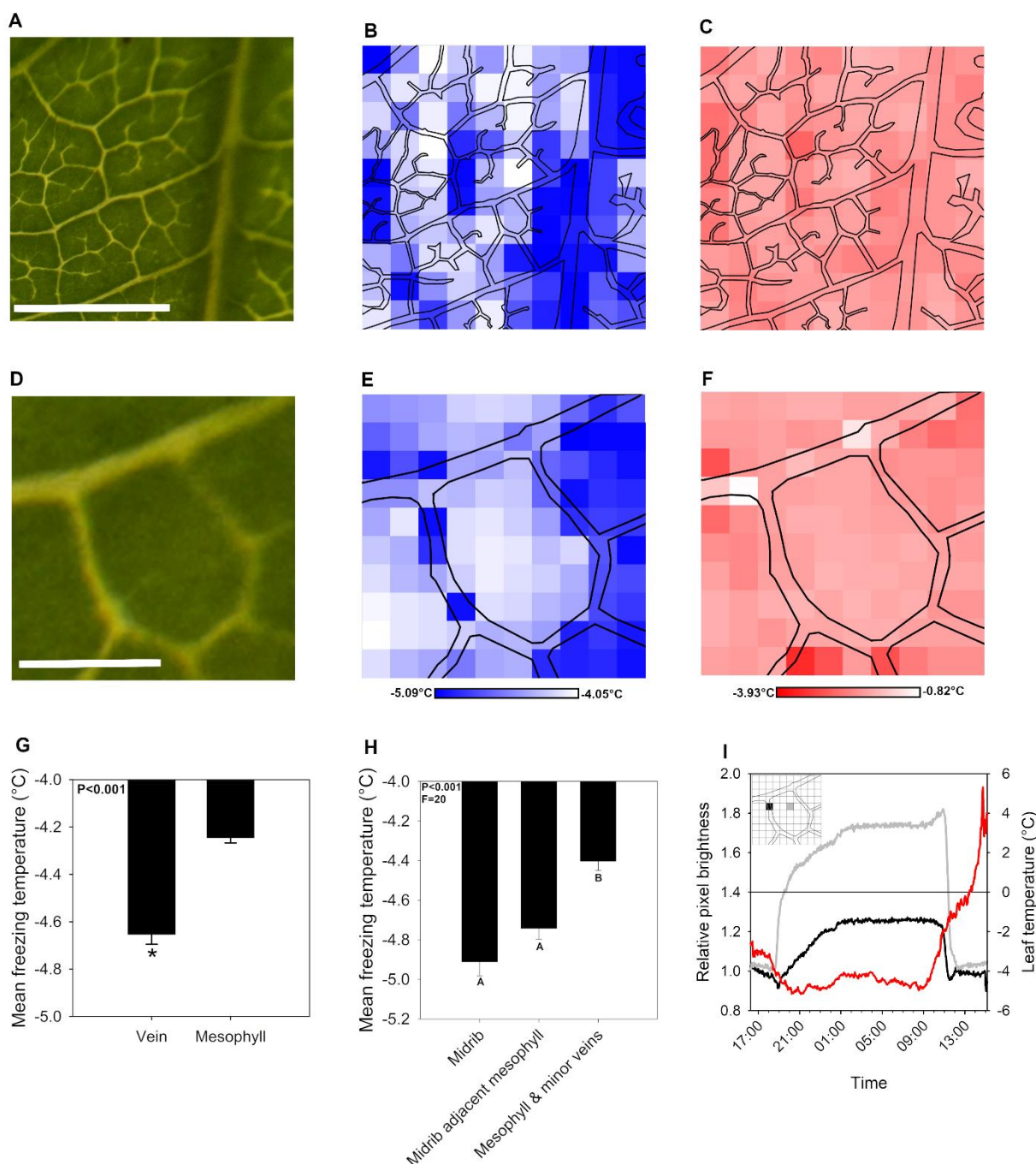


Figure 5-1. A. Shows a 450 by 450-pixel section of the first raw image of the image stack taken during a natural in-situ freeze thaw cycle of *Lonicera × purpusii*. The white scale bar indicates 2.5cm B. The 450 by 450-pixel section broken down into 45 by 45-pixel sections with each section colored based on the leaf temperature at which the mean pixel brightness increased by 10% from the initial value. With veins outlined in black. C. The same leaf section showing the leaf thawing temperature based on a decrease of 10% mean pixel brightness from maximum pixel brightness. D. Show a 90 by 90-pixel subsection of the first image from the raw image stack. The white scale bar is 0.5cm E. The 90 by 90-pixel section broken down into 9 by 9-pixel sections with each section colored based on the leaf temperature at which the mean pixel brightness increased by 10% from the initial value. With veins outlined in black. F. The same leaf section showing the leaf thawing temperature based on a decrease of 10% mean pixel brightness from maximum pixel brightness. G. The difference in mean freezing temperature between sections of the 90 by 90-pixel section that contain the veins and sections that contain the mesophyll surrounded by vein. The ‘\*’ indicates that they are significantly different based on a two-way student-T test. H. The difference in mean freezing temperature between sections of the 450 by 450-pixel section that contain the midrib, mesophyll adjacent to the midrib, and mesophyll and minor veins farther from the midrib. The letters indicates that they are significantly different based on a one-way ANOVA and a Tukey's HSD post hoc test (p<0.05). I. The time course during the night when freezing was measured out of relative pixel brightness of a 9 by 9-pixel section containing mostly vein (black) or areola (gray) with the leaf temperature (red). A horizontal line demarcated 0°C. The insert depicts the area the brightness was measured in with the black pixel being the vein and the gray pixel mesophyll.



## 5.4 Results

### 5.4.1 High resolution evaluation of leaf freezing using timelapse imaging

By converting pixels to brightness of a timelapse image stack taken of a leaf exposed to a natural freezing and thawing during a winter night, we were able to identify a clear transition between unfrozen, frozen, and then subsequently thawed leaf tissue, including in veins and mesophyll (Figure 5-1A; Supplementary Video 5-1). Analysis of the timelapse series of images identified a clear spatial pattern of freezing during the night, with the first pixels initiating freezing at  $-4.05^{\circ}\text{C}$  while the final pixels initiating freezing at  $-4.73^{\circ}\text{C}$  (Figure 5-1B). Freezing was initiated in the midrib at lower temperatures than the mesophyll and minor veins with mean midrib freezing occurring at  $-4.88^{\circ}\text{C} \pm 0.07$ , while freezing in the mesophyll and minor veins not adjacent to the midrib occurring at  $-4.37^{\circ}\text{C} \pm 0.05$  (Figure 5-1B). Mesophyll closest to the midrib froze at temperatures similar to those of the midrib, at  $-4.74^{\circ}\text{C} \pm 0.06$  (One way ANOVA,  $P < 0.001$ ,  $F = 20$ ) (Figure 1B). When a smaller  $1 \text{ mm}^2$  area of leaf spanning an areole not adjacent to the midrib was divided into 100 pixels and analyzed the freezing of pixels in the areole not intercepted by veins occurred at  $-4.24^{\circ}\text{C} \pm 0.01$ , while pixels intercepted by veins froze at a lower temperature of  $-4.65^{\circ}\text{C} \pm 0.04$  (Two way t-test,  $P < 0.001$ ) (Figure 5.1E). Thawing occurred at less negative temperatures than freezing occurred, between  $-2.92^{\circ}\text{C}$  and  $-1.72^{\circ}\text{C}$  (Figure 5-1C). No obvious spatial pattern was evident during thawing, at either high or low spatial scales (Figure 5-1C, Figure 5-1F, Supplemental figure 5-2B).

### 5.4.2 Effect of freezing speed on photosynthetic and water potential recover

Freezing speed had a significant effect (One way ANOVA  $P < 0.001$ ,  $F = 70.8$ ) on observed freezing temperature. In *ex situ* experiments leaves frozen at  $-1^{\circ}\text{C min}^{-1}$  and  $-10^{\circ}\text{C min}^{-1}$  froze at  $-2.32^{\circ}\text{C} \pm 0.49$  and  $-5.68^{\circ}\text{C} \pm 0.19$  respectively (Figure 5.2C) based on freezing exotherms (Figure 5-2B). While the *in situ* leaf froze at  $-4.58^{\circ}\text{C} \pm 0.035$  according to pixel brightness analysis (Figure 5-2C). Freezing exotherms in leaves frozen at  $-1^{\circ}\text{C min}^{-1}$  and  $-10^{\circ}\text{C min}^{-1}$  happen 1048 and 86 seconds after leaf temperatures dropped to  $0^{\circ}\text{C}$  with the actual release of latent heat of freezing taking 255 and 5 seconds respectively (Figure 5-2B). The leaf frozen *in situ* took approximately 4.4 hours to freeze completely (Figure 5.2A).

In *ex situ* experiments we found that leaves frozen at  $-1^{\circ}\text{C min}^{-1}$  could survive freezing to  $-10^{\circ}\text{C}$ , with only a minimal reduction (8.8%) in maximum assimilation rate measured on thawing (Figure 5-2D). In contrast, rapid freezing was found to permanently damage leaves exposed to temperatures that do not, or only minimally, damage photosynthetic capacity if frozen slowly (One way ANOVA  $P<0.001$ ,  $F=13.9$ ) (Figure 5-3). Gas exchange of thawed leaves that had been frozen at  $-10^{\circ}\text{C min}^{-1}$  was severely compromised, with recovered assimilation rate being 11.7% of maximum ( $n=6$ ), (Figure 5-2D). We found that leaves *in situ* naturally frozen at  $-0.01^{\circ}\text{C min}^{-1}$  ( $n=3$ ) had similar recovery to those frozen at  $-1^{\circ}\text{C min}^{-1}$  with reductions in assimilation also of 8.8% (Figure 5-2D). Leaf water potential was not significantly affected by rate of freezing (One way ANOVA  $P=0.47$ ,  $F=0.8$ ) (Figure 5-2E).

*Ex situ* freezing at  $-1^{\circ}\text{C min}^{-1}$  resulted in similar freezing temperatures of leaf ( $-2.32^{\circ}\text{C} \pm 0.49$ ) and exposed stem xylem ( $-2.14^{\circ}\text{C} \pm 0.5$ ) while xylem sap ( $-0.36^{\circ}\text{C} \pm 0.084$ ) froze at a significantly lower temperature (One-way ANOVA,  $P=0.026$ ,  $F=7.1$ ) (Supplemental figure 5-2A)

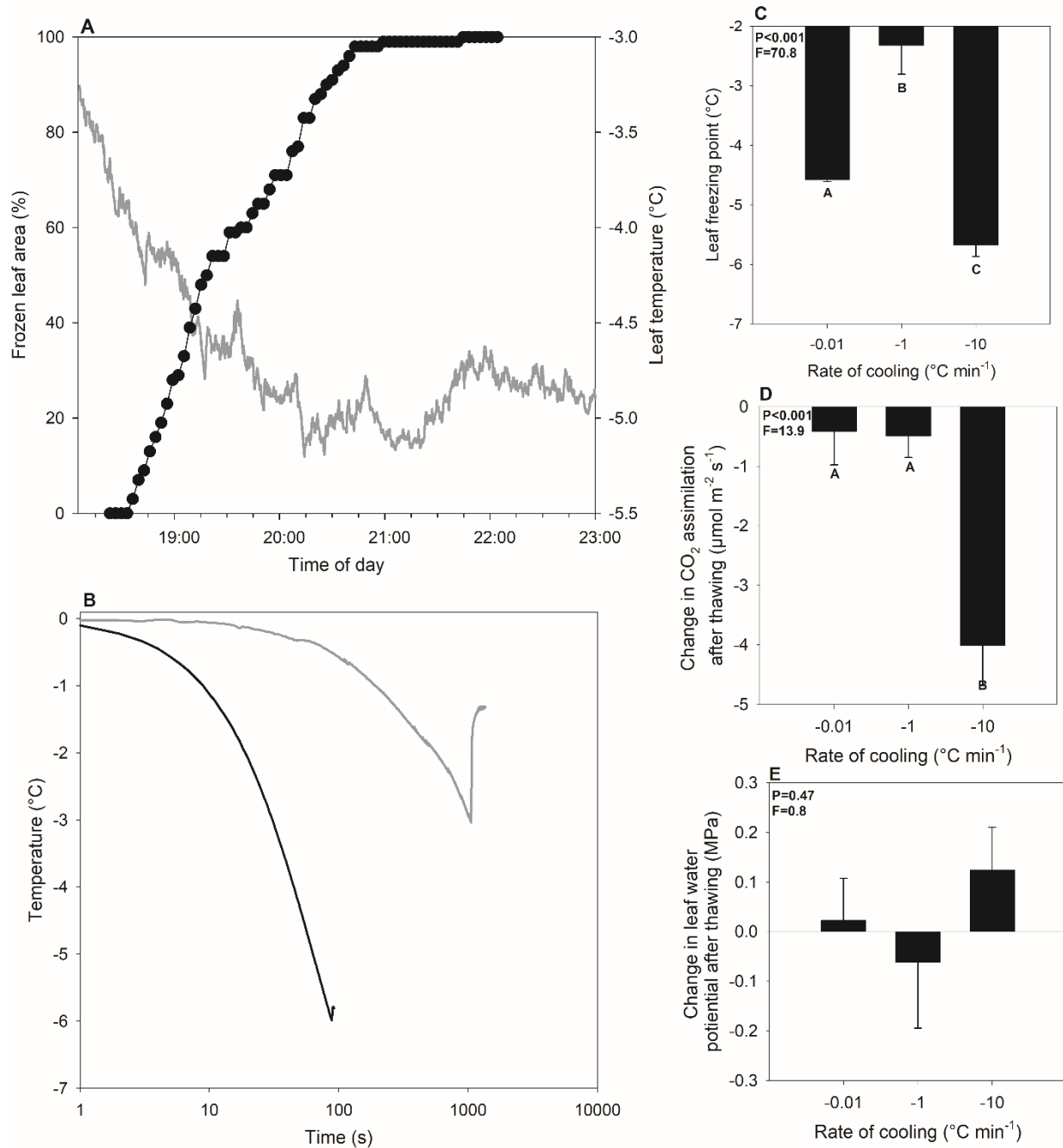


Figure 5-2. A. The percent of leaf pixels that have initiated freezing (black) over the course of the night of DOY 28 and the raw leaf temperature on the same night (gray). B. Freezing exotherms of *ex situ* leaves frozen at  $-1^{\circ}\text{C min}^{-1}$  (gray) and  $-10^{\circ}\text{C min}^{-1}$  (black) vs the time in seconds since the leaves reached  $0^{\circ}\text{C}$ . C. Freezing temperatures of leaf frozen at  $-0.01^{\circ}\text{C min}^{-1}$ ,  $-1^{\circ}\text{C min}^{-1}$ , and  $-10^{\circ}\text{C min}^{-1}$  with standard errors. Letters denote a significantly different mean based on a one-way ANOVA and a Tukey's HSD post hoc test ( $p < 0.05$ ). D. Photosynthetic recovery of leaves frozen at  $-0.01^{\circ}\text{C min}^{-1}$ ,  $-1^{\circ}\text{C min}^{-1}$ , and  $-10^{\circ}\text{C min}^{-1}$  with standard errors. Letters denote a significantly different mean based on a one-way ANOVA and a Tukey's HSD post hoc test ( $p < 0.05$ ). E. Change in leaf water potential of leaves frozen at  $-0.01^{\circ}\text{C min}^{-1}$ ,  $-1^{\circ}\text{C min}^{-1}$ , and  $-10^{\circ}\text{C min}^{-1}$  with standard errors. Letters denote a significantly different mean based on a one-way ANOVA and a Tukey's HSD post hoc test ( $p < 0.05$ ).

### 5.4.3 Insulation by snow can protect leaves from severe freezing events.

Leaves in the field that had been exposed to minimum temperatures of  $-19^{\circ}\text{C}$  in the air did not recover photosynthetic rate when thawed, compared to leaves of the same plant buried under snow (Figure 5-3). Warmed leaves that had been exposed to cold air respired, while leaves buried under snow were capable of positive assimilation rates with a mean of  $2.43 \text{ mmol m}^{-2} \text{ s}^{-1} \pm 0.67 \text{ } \mu\text{mol m}^{-2} \text{ s}^{-1}$  ( $n=3$ )  $p=0.0151$  (two-tail t-test) measured at midday. Low leaf water potentials were not the cause of photosynthetic failure in leaves exposed to the air, which had an average leaf water potential of  $-0.80 \pm 0.64 \text{ MPa}$  ( $n=3$ ), similar to the water potential of leaves buried under snow  $-0.57 \pm 0.28 \text{ MPa}$  ( $n=3$ )  $p=0.5994$  (two-way t-test).

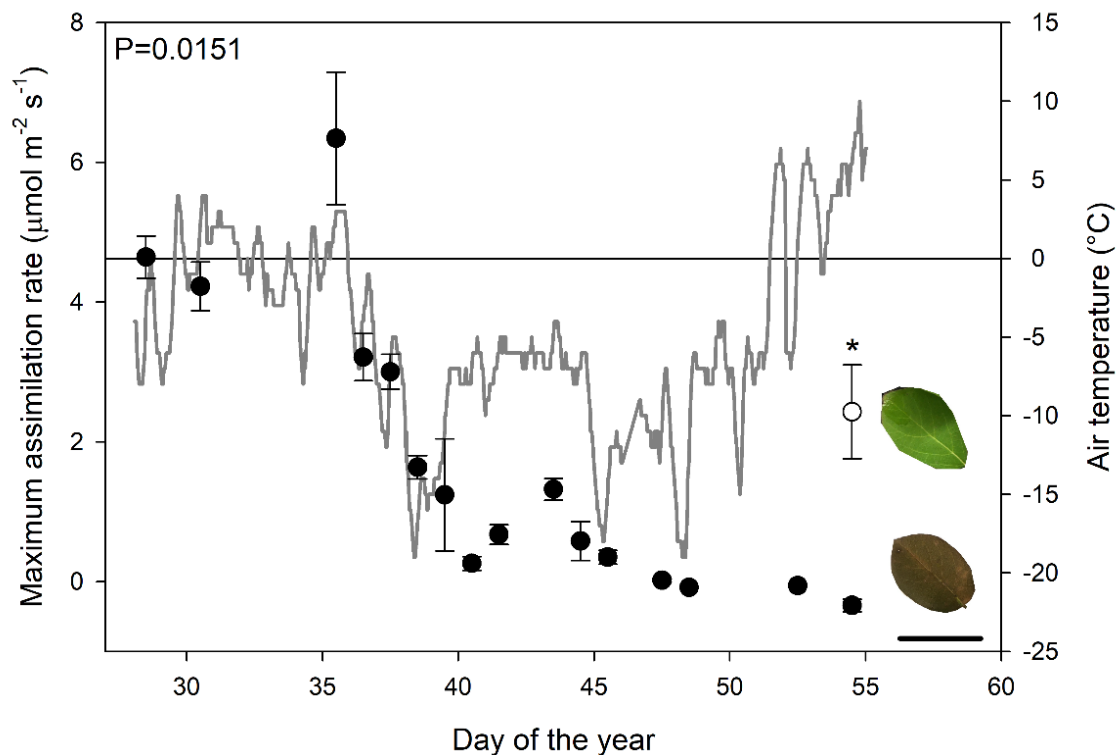


Figure 5-3. The mean maximum assimilation rate of leaves measured at  $22^{\circ}\text{C}$  that had been exposed to air during the snow storm and cold period (Black points), the white point represents the mean maximum assimilation rate measured on leaves from a branch that was buried under snow from the onset of the snowfall until the day of measuring ( $n=3$ ,  $\pm\text{SE}$ ). The gray line depicts air temperature. The '\*' denotes a significant difference between the maximum assimilation rates of the leaves exposed to air and buried under snow DOY 54.

## 5.5 Discussion

We demonstrate a low cost, easily accessible method for capturing the pattern and timing of freezing and thawing in leaves using timelapse photography. This is similar to the earlier work of Kaku, (1971) who, at a much lower spatial scale was able to determine the initiation point of leaf freezing. We used pixel brightness as a proxy for leaf freezing, a method that is similar to the satellite determination of sea ice flows (Roach, Smith & Dean 2018). Using this method, we observed that the midrib and the mesophyll that surround it freeze at the lowest temperatures in *L. × purpusii* with mesophyll and minor veins being the first tissues to freeze. A similar spatial pattern of initial mesophyll freezing, most distant from the major veins, has been observed in *Senecio incanus* and some leaves of *Buxus microphylla* (Kaku 1971; Hacker, Spindelböck & Neuner 2008; Hacker & Neuner 2008). The process by which ice forms and spreads in leaf tissue appears to be species dependent and can begin in the midrib in some species, particularly conifers (Hacker & Neuner 2008). In *Pinus mugo* needles freeze in the midrib and the endodermis acts to keep ice from spreading to the mesophyll (Stegner *et al.* 2023). Other determinants of the tissue of initial freezing include leaf age, size, and water content (Kaku 1971). Leaves that supercool during sub-zero air temperatures maintain leaf water that is still liquid below 0°C, only once ice nucleation beings at a nucleation site can the ice spread through the leaf. The number of nucleation sites varies depending on leaf anatomy, microbial colonization, and other unknown factors (Kaku 1975; Wisniewski, Lindow & Ashworth 1997; Hacker & Neuner 2008). Our new method provides a quick and highly affordable means of examining the spatial freezing and could be deployed to address these key unknowns about the spatial and temporal dynamics of tissue freezing and thawing.

In *L. × purpusii* we observed that the veins froze at the lowest temperatures, to test whether xylem sap has innately lower freezing points than leaves we extracted xylem sap and determined the freezing point (Supplementary Figure 5-2A). We found that when xylem sap is extracted and frozen outside of the plant the freezing temperature is almost 0°C. With very little of the radial and axial volume in xylem tissue occupied by living parenchyma (Spicer 2014), even in the weaker wood of *Lonicera* species (Ogata 1988), most of the volume of water *in situ* in the stem is assumed to be in xylem cells. This difference in temperature between the freezing point of xylem and xylem sap suggests that some unknown aspect of water contained in vessel elements depresses the freezing point. More work is needed to address the question of why water under mild tension in

vessels might be more resistant to freezing than that same water extracted from the conduits, or in the case of intact leaves more resistant to freezing than the water of mesophyll cells. Some studies have found correlations between vessel size and minimum freezing temperatures (Cavender-Bares *et al.* 2005), suggesting that the vascular anatomy of a given species may play an important role in determining freezing temperatures of the xylem. It is thought that the water in narrower vessels is more capable of reabsorbing expelled gas (expelled on freezing) when thawing (Sperry & Sullivan 1992; Utsumi *et al.* 1999). It has also been suggested that differences in freezing damage based on vessel size maybe due to larger vessels freezing at less negative temperatures than smaller vessels leading to larger vessels being embolized by freezing (Lo Gullo & Salleo 1993; Cavender-Bares *et al.* 2005). Our new method for determining freezing dynamics and timing could be used to confirm possible relationships between xylem anatomy and freezing temperatures, and at a higher spatial scale could have the resolution for identifying the freezing points of individual conduits, much like timelapse photography can reveal the embolism thresholds of near- conduit level resolution.

Our work shows that freezing speed is critical for the survival of *L. × purpusii* tissue. The ability of leaves to recover gas exchange capacity on thawing and also surviving long periods frozen under snow suggests that the xylem of frost tolerant *L. × purpusii* did not experience freeze thaw embolism, which would have damaged the water transport stream (Skelton, Brodribb, McAdam & Mitchell 2017; Cardoso, Batz & McAdam 2020). Fruthermore, our timelapse analysis did not detect any evidence of embolism forming in leaf veins in leaves exposed to a natural freezing and thawing cycle in *L. × purpusii*. Freezing speed also appeared to have a major impact on the observed freezing temperature of leaves. With three different cooling rates yielding three significantly different freezing temperatures (Figure 5-2C). Rapid cooling can cause an incorrect determination of bulk tissue freezing, either by snap freezing giving a underestimate (Pearson & Davison 1993) or the rapid cooling will cause supercooling faster than in nature leading to and overestimate of freezing temperature (Salt 1966). When designing experiments to look for frost tolerance, survival, and tissue freezing temperature a slower cooling rate is preferable.

*L. × purpusii* leaves can recover function through repeated mild freezing events in a season with photosynthesis recovering on thawing, even at very low air temperatures (Figure 5-3). *L. × purpusii* leaves *in-situ* were able to survive minimum nighttime temperatures above -19°C with minimal damage to maximum photosynthetic capacity. Once leaves were frozen below -19°C upon

thawing tissue would turn brown, and photosynthesis was unrecoverable. Lower branches buried in snow during the coldest days of winter were protected, remaining green on thawing and could still undertake photosynthesis. This color change in damaged leaves upon thawing may mean that extending time lapse analysis may also offer insights into damage leaves experienced during freeze thaw cycles. Snow is well recognized as an insulator against severe air freezing (Neuner, Ambach & Aichner 1999; Taschler & Neuner 2004; Briceño, Harris-Pascal, Nicotra, Williams & Ball 2014).

Despite the climate generally warming unusual frost events may become more common (Lamichhane 2021). Warmer temperatures will cause deciduous plants will leaf out earlier putting them at risk for late spring frosts (Menzel *et al.* 2015; Zohner, Mo & Renner 2018) and the slowdown of the Atlantic meridional overturning circulation may cause much cooler temperatures in Europe (Jackson *et al.* 2015; Ditlevsen & Ditlevsen 2023) posing new freezing risks to large sections of deciduous forest. Our timelapse imaging technique is an affordable and simple method for observing freezing patterns and freezing initiation sites in leaves *in situ*. This method could be used to monitor natural freezing events in the field. Like the optical vulnerability method for determining embolism resistance from image subtraction which has greatly increased the number of species for which we now have an ever-growing dataset of key water potentials of mortality (Cardoso, Kane, Rimer & McAdam 2022), our new method provides a simple means of widely sampling freezing tolerance thresholds across species which could improve our modelling of forest and community responses to aseasonal freezing events and more accurately inform models of range changes into the future (Tranquillini 1982; Inouye 2000; Walker *et al.* 2004; Stuart *et al.* 2007; Löffler 2007). Our work also shows that when evaluating frost survival *ex situ* it is critical to account for freezing speed as rapid freezing can cause damage to leaves that may have survived if frozen slower.

## 5.6 Acknowledgements

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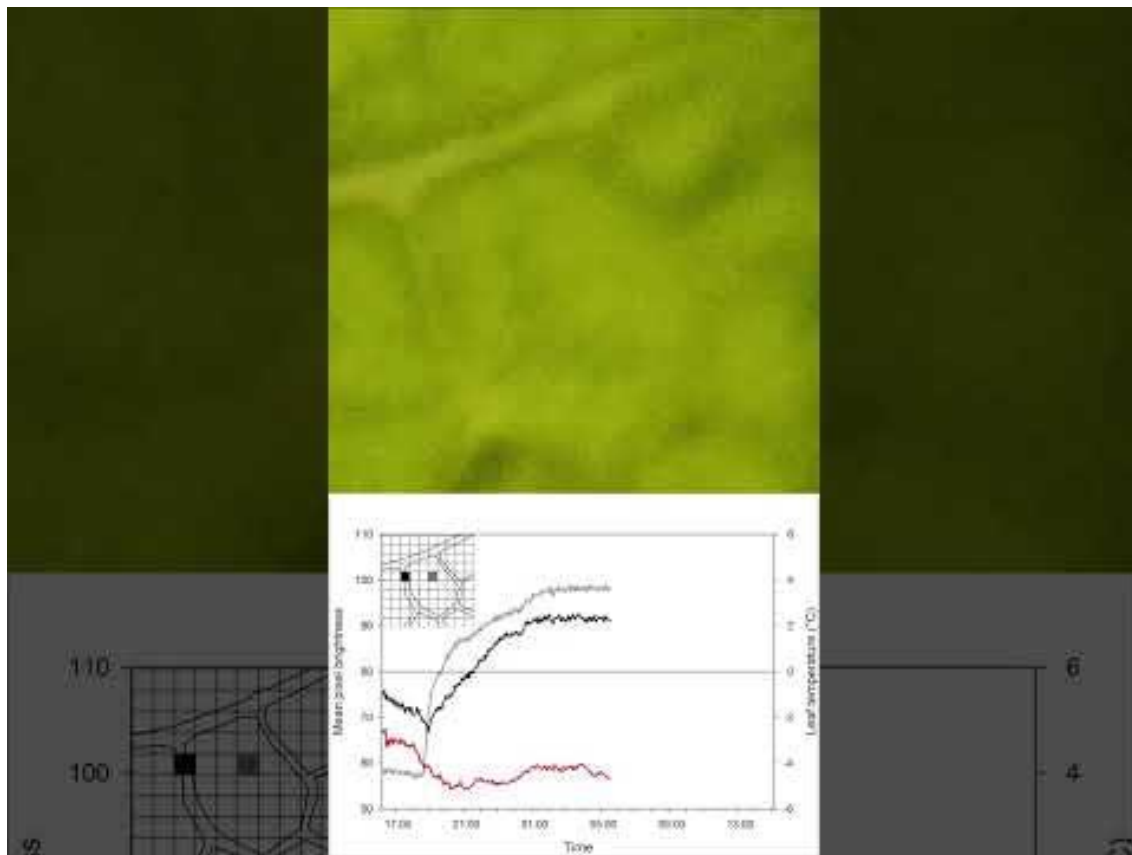
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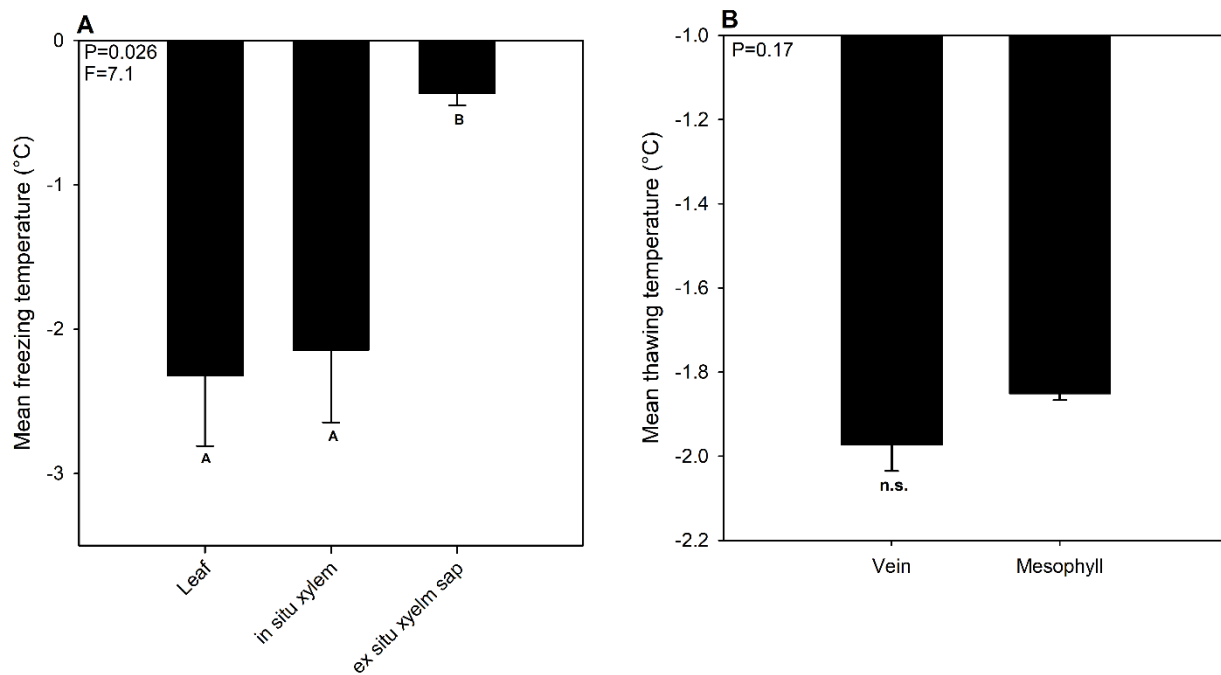
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## 5.8 Supplemental material



Supplemental video 5S.1. Video depicting the raw time lapse of the 90 by 90-pixel section freezing and thawing DOY 28 2021. The figure below shows the time course during the night when freezing was measured total pixel brightness of a 9 by 9-pixel section containing mostly vein (black) or areola (gray) with the leaf temperature (red). A horizontal line demarcated 0°C. The insert depicts the area the brightness was measured in with the black pixel being the vein and the gray pixel mesophyll.



Supplemental figure 5.S2. A depicts the mean (n=3) freezing temperature of *ex situ* leaves, exposed xylem tissue, and extracted xylem sap the letters denote significant differences in means based on a one-way ANOVA and a Tukey's HSD post hoc test ( $p < 0.05$ ). B. The difference in mean thawing temperature between sections of the 90 by 90-pixel section that contain the veins and sections that contain the mesophyll surrounded by vein. The 'n.s.' indicates that they are not significantly different based on a two-way student-T test.

## CHAPTER 6. HIGH PHENOLOGICAL DIVERSITY BLURS THE PREDICTIONS OF FUTURE FOREST RESPONSES TO CLIMATE CHANGE

The text and results of this chapter are under review at *Science*:

### 6.1 Abstract

Zohner *et al.* (Research Articles, 7 July 2023, Vol 381, Issue 6653) proposes that warmer temperature in the spring and autumn will have opposing effects on leaf lifespan in deciduous trees. This analysis did not consider the high diversity of phenological triggers and responses to warmer temperatures that exist in deciduous tree species.

### 6.2 Main text

Zohner *et al.* 2023 recently conclude that a warming atmosphere across the year will have no net change on leaf life span in deciduous forests. This is an alarming discovery as prevailing data had indicated the growing seasons of forests were lengthening, increasing the terrestrial sink for atmospheric CO<sub>2</sub>, and without drought would continue to lengthen (Menzel & Fabian 1999; Norby, Hartz-Rubin & Verbrugge 2003). We argue that this recent synthesis fails to recognize the breadth of mechanistic diversity in deciduous tree phenology, a diversity that has far-reaching ramifications for the ecology and carbon capture of these forests over the next century and beyond.

Deciduous tree phenology is highly diverse, primarily driven by temperature and day length, with the relative importance of these two factors being a function of genotype, latitude and altitude of origin (Vitasse *et al.* 2013; Peaucelle *et al.* 2019). Consequently, profound differences in the phenological responses to warming can be observed between co-occurring species even from the same family. For example, species of oak (*Quercus*) have been found to have a lengthening growing season under higher temperatures (Vitasse *et al.* 2011) which contrasts with species of beech (*Fagus*) (both Fagaceae) which exhibit strong photoperiodism and little phenological shift in response to similar increases in temperature ((Vitasse *et al.* 2011; Zohner, Benito, Svenning & Renner 2016); Figure 6-1C and E). Our comparisons of the leaf life span of genetically identical individuals grown in a common latitude experiment highlight this diversity in response to future



warming, with few of our observed phenological differences able to be explained by the new model of Zohner *et al.* 2023; Figure 6-1). While equidistant from the equator the two sites in which these clones grew differed in cold-season temperature regime by a margin that exceeds the worst-case scenario for future climates across Northern temperate forests (Figure 6-1). We observed clones of English oak (*Q. robur* ‘Fastigiata’) burst bud 59 days earlier in the warmer spring and retained leaves 39 days later in the autumn at the Southern Hemisphere site, showing a prolonged leaf lifespan before and after the summer solstice in response to warmer winter temperatures (Figure 6-1C). This contrasted with clones of London plane (*Platanus x acerifolia*) which burst bud 56 days earlier in the Southern Hemisphere site but retained leaves 6 days longer in the Northern Hemisphere site, indicating that this species has a temperature-responsive bud burst and a photoperiodic-dominated regulation of autumnal senescence (Figure 6-1D). Clones of European beech (*F. sylvatica* f. *purpurea*) were found to have invariant phenology regardless of the temperature regime of the two sites (Figure 6-1E). We argue that capturing this considerable interspecific diversity in phenological response to warming is critical for models of future forest phenology. The phenological diversity that exists in temperate forests is likely to have major and imminent impacts on species competition and forest composition as the climate continues to warm (Vitasse *et al.* 2011, 2013). The much longer growing season afforded English oak under warmer temperatures compared to the absent response of the photoperiodic-regulated and co-occurring European beech may lead to rapid ecological transitions in Europe’s oak-beech forests in the coming century ((Vitasse *et al.* 2011; Zohner *et al.* 2016); Figure 6-1C and E), an outcome absent from the future projections of forest phenology proposed by Zohner *et al.* 2023

Despite the likelihood of diverse seasonal phenology, Zohner *et al.* 2023 found that over the past 17 years when there were warm springs and early bud burst there was an advanced autumnal senescence that was attributed to a faster annual accumulation of carbon stores. However, key metrics of water stress, which can lead to early senescence in deciduous trees (Frei *et al.* 2022), were not incorporated in this analysis raising the possibility that water deficit (atmospheric or soil) rather than carbon balance may have been the driver of these phenological trends. Zohner *et al.* 2023 binned water deficit into two categories: total precipitation from the vernal equinox to the summer solstice; or precipitation for the remainder of leaf lifespan after the summer solstice. Precipitation metrics do not incorporate vapor pressure deficit (VPD) and potential evapotranspiration (PET) which are more predictive drivers of leaf water status (Williams *et al.*

2012; D'Orangeville *et al.* 2018). Even when precipitation is consistent across years, increasing VPD driven by higher temperatures will lead to higher PET (D'Orangeville *et al.* 2018; Gamelin *et al.* 2022); earlier bud burst combined with higher PET will more rapidly deplete soil water reserves, such that by the summer solstice increased water deficit may have occurred in each of the years of a warm spring triggering an earlier autumnal senescence. The use of only spring and summer precipitation also neglects winter precipitation and the importance of winter recharge of ground water reserves. Winter precipitation has been shown to have an influence on spring phenology and in some forests water deposited in the cold season is the primary water source transpired during the growing season (Yun *et al.* 2018; Huang *et al.* 2022).

Biotic factors, and not phenology, could have also explained the observations of earlier leaf loss, or declines in canopy greenness, in years with warmer springs in this new model (Zohner *et al.* 2023). Increased insect and disease damage to the canopy in years with warmer springs may explain the early declines in green reflectance which was used as a proxy for autumnal senescence (Zohner *et al.* 2023). Warming temperatures are increasingly allowing insects and diseases to invade habitats previously unoccupied (Anderegg *et al.* 2015). Warmer seasons are also causing, now well-documented, earlier outbreaks of insect populations that can defoliate canopies (Anderegg *et al.* 2015). While warmer winters and springs in particular lead to increases in plant pathogen and insect loads, with cold temperatures reducing overwintering populations (Weed, Ayres & Hicke 2013). Consequently, early senescence could have been mistakenly assigned to warmer years with higher insect or pathogen loads.

Considerable diversity in the temperature regulation of leaf life span, combined with an unknown influence of abiotic and biotic drivers of canopy health, weakens our current ability to accurately forecast future deciduous forest phenology. We would argue that this shortfall must be addressed urgently through an increase in the number of studies characterizing phenological plasticity and diversity across deciduous species, especially if we wish to have any chance of accurately predicting future phenology, carbon sequestration and ecology of deciduous forests into the next century.

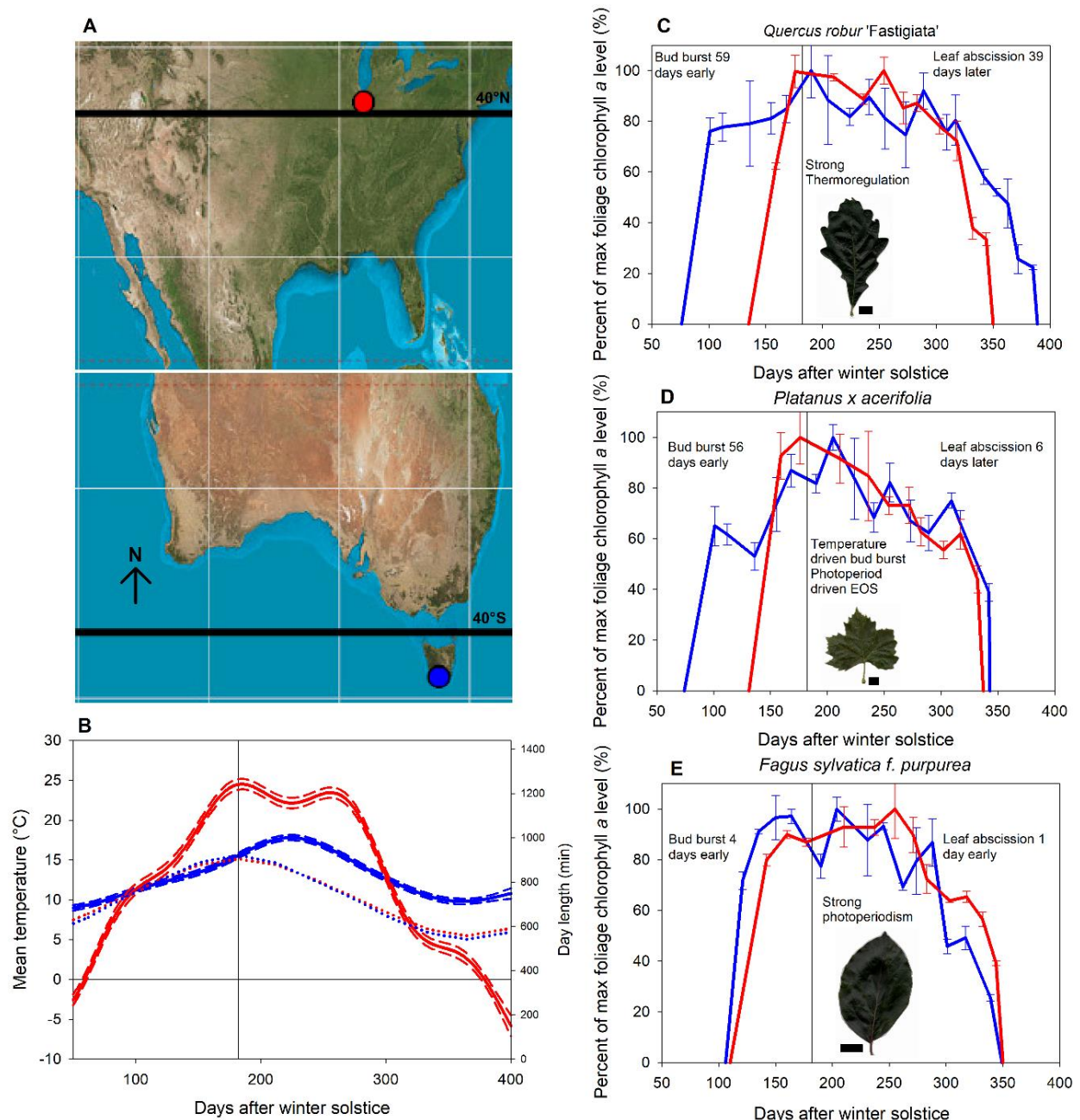


Figure 6-1. Considerable diversity in the phenological response to warmer climates exists across canopy dominant, temperate deciduous forest species. (A) In a common latitude experiment in which phenology was tracked across a year in clones of three deciduous tree species grown at sites equidistant from the equator in West Lafayette, Indiana, USA (red) and Hobart, Tasmania, Australia (blue) a wide diversity of phenological responses were observed. (B) These sites have similar daylength during the growing season (dotted lines), yet daily mean temperature differs dramatically, with temperature data from both West Lafayette (red) and Hobart (blue) fitted with a general additive model and standard error (dashed line) for the growing season in the years of data collection (West Lafayette in 2021 and Hobart (2022-2023)), the vertical line denotes the summer solstice, while the horizontal line marks 0°C. Mean percent of maximum leaf chlorophyll *a* content ( $n=3$ ,  $\pm$ SE) for clones of (C) *Quercus robur* 'Fastigiata', (D) *Platanus x acerifolia* and (E) *Fagus sylvatica* f. *purpurea* grown in West Lafayette (red) and Hobart (blue). The first points represent the day of bud burst and the final point the day of complete leaf loss, the vertical line denotes the summer solstice. All dates are relative to the winter solstice at each site. A representative leaf of each species is shown (scale bar = 2cm).

### 6.3 Acknowledgments

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### 6.4 References

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## CHAPTER 7. CONCLUSIONS AND FUTURE WORK

Despite the significance of deciduous tree phenology to various cultures in the northern and southern hemispheres, our understanding of the physiological and environmental drivers behind spring leaf-out and autumn senescence/abscission is limited (Xie, Wang, Wilson & Silander 2018). The phenology of temperate trees also contributes billions of dollars to tourism each year (Sandifer, Sutton-Grier & Ward 2015). From the spring cherry blossoms in Japan and Washington D.C. to global leaf peepers who annually flock to forested areas to observe the dynamic autumn foliage. Winter deciduous processes are present in most major plant groups, including ferns, gymnosperms, and angiosperms, with lycophytes exhibiting some drought deciduous phenotypes in certain species (Gower & Richards 1990; Killingbeck, Hammen-Winn, Vecchio & Goguen 2002; Gonzatti, Valduga, Wasum & Scur 2014). Deciduous traits have likely evolved multiple times throughout plant evolution (Edwards *et al.* 2017). These evolutions have likely been caused by long stretches of inhospitable environmental conditions which may have been one or multiple environmental pressures, like long stretches of minimum sunlight that are found at the poles (Wolfe & Upchurch 1986). This strategy of avoiding dark periods is still in use today by spring ephemerals that will leaf out early then senesce to avoid shade by the upper canopy trees (Augspurger & Salk 2017). The Asian shrub *Daphne pseudomezereum* which in Japanese forests have become summer deciduous where leaf out occurs in the autumn the plants will then photosynthesize during the winter and spring then lose its leaves in the summer to avoid shading by upper canopy trees, this strategy appears to be reality derived as there is a variety in Korea that has the much more typical winter deciduous phenology (DaHyun, HoJun, SungHyuk, SeChang & WanGeun 2019; Lei 2020). Another pressure that likely led to deciduous evolution was seasonal droughts (Chabot & Hicks 1982; Edwards *et al.* 2017). Leaf loss at the onset of drought is a characteristic observed annually in drought deciduous species like *Erythrina sandwichensis* do annually to conserve water and avoid damaging embolism, these species will quickly leaf out again after rains return allowing for continued photosynthesis (Choat *et al.* 2012; Hochberg *et al.* 2017; Cardoso, Batz & McAdam 2020a; Wagner, Herbst & Sohmer 671–672). The final evolutionary track that appears most parsimonious to people currently living near winter deciduous forests is that plants evolved this habit to avoid long cold winters, reducing photosynthesis while also putting the plant at an increased risk of experiencing damaging frosts (Zanne *et al.* 2014; Edwards *et al.*

2017). Although my thesis has expanded our understanding of the secret lives of deciduous trees, it is by no means exhaustive. There are many areas of deciduous tree phenology and physiology that remain understudied, or presumptions drawn from work done on herbs and crop plants are assumed to carry over to longer lived species. One primary observation in my thesis is to avoid presuming that what has been learned in *Arabidopsis* and other model species cannot always be applied to trees, either wholly or partially.

## 7.1 Water, stomata, and expanding leaves

Plants are constantly losing water from every above ground surface at different rates. Understanding the extent of water loss from different surfaces is crucial for determining which trees are best equipped to survive future droughts. In drought conditions, if plants close their stomata, water will still be lost from incompletely closed stomata, cuticle, and bark (Duursma *et al.* 2019; Wolfe 2020). If a tree has higher water loss through the bark or cuticle, then neighbors they may have higher risks of embolism and injury from drought (Wolfe 2020). Understanding this is crucial for developing leaves, as they are most vulnerable to damage during spring droughts (D'Orangeville *et al.* 2018). To comprehend the paths and properties of water loss, I contributed to measuring water loss in *Fagus gradifolia* and *Tilia americana*, representing anisohydric and isohydric species, respectively, from bark, cuticle, and stomata (Baker *et al.* 2023: Under review *New Phytologist*). We found that minimum conductance was highest in both species early on then drops off quickly after as leaves expand (Baker *et al.* 2023: Under review *New Phytologist*). Additionally, our findings reveal that most water is lost through the cuticle until stomata develop and form their outer cuticular ledge (Baker *et al.* 2023: Under review *New Phytologist*). This confirms my observations on water loss in expanding leaves in *Quercus rubra* (Chapter 2). Our discovery indicates that bark conductance is somewhat significant during leaf expansion, but its importance diminishes once leaves are fully expanded (Baker *et al.* 2023: Under review *New Phytologist*). I aim to continue my research on expanding leaves. I want to be able to use scanning electron microscopy to investigate the origin of the stomatal cuticular covering by looking at the development of leaves from all major plant groups including mosses capsules, which are the evolutionary origin of stomata. It would also be interesting to investigate the causes of cuticle tearing; I proposed mechanical force in Chapter 2 while it has also been suggested to be enzymatic breakdown of the cuticle covering (Carr & Carr 1978). The model proposed by Pantin *et al.* 2013

for *Arabidopsis*, suggesting stomata are present on extremely young leaves, wide open at inception, and insensitive to ABA until exposure to dry air, does not apply to *Q. rubra*, which lacks stomatal pores until almost fully expanded (Chapter 2). I also observed a similar stomatal ontogeny in developing leaves of *Arabidopsis Col-0* (Chapter 2).

## 7.2 Drought, heat, and ABA

Climate change is making droughts more frequent and more severe and with this forest mortality is increasing. Droughts lead to tree mortality by intensifying tension on the water in the xylem, eventually causing the water column to break, forming an unrecoverable embolism (Allen *et al.* 2010; Cardoso *et al.* 2020a). This embolism can cause reduced photosynthetic capacity, partial leaf death, or even whole plant death (Brodribb & Cochard 2009; Choat *et al.* 2018). In seed and flowering plants, it is known that Absciscic acid (ABA) disrupts ion transport into stomatal guard cells, causing them to lose turgor and close (MacRobbie 1995; Susmilch, Brodribb & McAdam 2017). This closure reduces water loss from the leaves, slowing the build-up of tension in the xylem (Hochberg *et al.* 2017; Creek *et al.* 2020). ABA is not commonly measured during droughts and drought experiments, due to this it is occasionally forgotten or discounted as an important protector of plants during drought (Leuschner 2020; Leuschner, Schipka & Backes 2022). This is particularly true for anisohydric species, which are characterized by stomata that are less responsive to drought, with much smaller hydraulic safety margins (Sade & Moshelion 2014; Hochberg, Rockwell, Holbrook & Cochard 2018; Leuschner *et al.* 2022). I observed in Chapter 3 that during a natural drought in the anisohydric species *Fagus sylvatica* ABA levels increase as water potential declines, and this corresponds with reductions in stomatal conductance. This indicates that anisohydric angiosperm trees are probably using ABA to close stomata during drought. This closing of the stomata also leads to a stabilization of the leaf water potential which protected the leaves from embolism as the leaves did not approach the p50 where 50% of the xylem elements would be filled with air. It also aligned with work done indicating that the trigger for ABA production corresponds with turgor loss in leaves (Bacete *et al.* 2022).

I am eager to investigate more species classified as isohydric vs anisohydric and conduct controlled drought experiments to understand how ABA dynamics differ between the two groups. This includes expanding on previous work in *F. sylvatica*, examining the continuum of an/isohydric responses in individual species to determine if these responses are influenced by local



rainfall or other environmental factors (Nguyen, Polle & Pena 2017). I also hope to investigate ABA response in the genus *Celtis*. I have observed during a natural drought in a forest that leaves of *C. occidentalis* will dehydrate past the point of embolism formation while maintaining open stomata (Kane *et al.* 2023; In prep). There is a suggestion that *Celtis* evolved its deciduous habit from subtropical dry forests, radiating upwards and shifting from a drought to a winter deciduous habit (Edwards *et al.* 2017). I hypothesize that winter deciduous *Celtis* may develop insensitivity to ABA as leaves age, intentionally drying leaves to senesce and abscise early in the case of a drought. It could also be possible that *C. occidentalis* is a naturally occurring widespread ABA production mutant. If this is the case, I want to investigate whether this is a widespread trait among the genus *Celtis*, including those species that still grow in the tropics and subtropics.

In addition to drought high heat is another risk to tree life and forest mortality (Allen *et al.* 2010). Heat waves are also predicted to increase in severity with climate change (Marx, Haunschild & Bornmann 2021). When heat is combined with dry air, it elevates the vapor pressure deficit (VPD) to levels capable of rapidly extracting enough water from the leaves, leading to stomatal closure or even embolism in the leaf (Schultz & Matthews 1997; Cardoso, Brodribb, Kane, DaMatta & McAdam 2020b). The waxy cuticle layer that shields leaves from desiccation has a melting point (Hess & Foy 2000). I am interested in investigating changes in cuticle conductance ( $g_{\text{cute}}$ ) and minimum stomatal conductance when plants are exposed to high heat. Additionally, testing to determine if the deformation of the cuticle after high heat exposure is repairable once it returns to ambient temperatures. Cuticles are known to self-assemble on leaf surfaces; thus, it may be the case that they will correctly reassemble after melting in a heatwave. If trees undergo heatwaves that damage their cuticle, allowing them to become leakier, this could severely worsen water relations for that tree throughout the remainder of the season, potentially limiting carbon acquisition (Schreiber 2005).

### **7.3 Senescence, abscission, and leaf death**

Leaf senescence is a critical part of deciduous leaf phenology when nitrogen and other mobile nutrients are pulled back from leaves and stored in permanent tissues for use next season (Lim, Kim & Gil Nam 2007). This seasonal shift in nutrients includes nitrogen contained in chlorophyll. These declines in chlorophyll reveal other pigments like carotenoids turning leaves from green to yellow (Roberts 1937). Some plants like species of *Acer* and *Quercus* will produce

red anthocyanins that turn the fall leaves red or orange (Roberts 1937). Anthocyanins are thought to protect the leaf from sunlight during the senescence process (Feild, Lee & Holbrook 2001). When leaves change color every autumn thousands flock out of cities to get a chance to see the vivid autumn colors. It also provides an opportunity for people living in more developed, higher latitudes to connect with and appreciate nature. It has even inspired holiday home rental agencies to create their own fall foliage maps (<https://smokymountains.com/fall-foliage-map/>). This process is also common in herbs and annual plants although this results in whole plant death rather than the death of an organ but the continuation of the whole plant as is common in deciduous plants (Lim *et al.* 2007). The process of senescence is highly complex and can vary significantly between leaves of longer-lived trees and those of annual plants (Lim *et al.* 2007). Lots of work has been done to investigate the triggers and causes of leaf senescence in herbs and crop plants like *Arabidopsis* and rice (Liang *et al.* 2014; Gao *et al.* 2016; Liu, Longhurst, Talavera-Rauh, Hokin & Barton 2016). This is a major area I have wanted to interrogate in my thesis, to test assumptions about senescence in herbs to see if they do apply in deciduous species. In Chapter 4, I investigated whether ABA levels in deciduous leaves increase during the senescence process and if artificially increasing those levels by girdling the branch would accelerate senescence. I discovered that there was no natural increase in foliar ABA levels during normal senescence, but that if a branch is girdled ABA levels will increase in two of the species leading to accelerated senescence in the autumn. Two of the girdled species did not show increases in foliar ABA levels and no change in senescence rate. This suggests that changes in ABA levels were a significant factor driving observed changes in leaf senescence rates. ABA has been shown to increase during the very end of leaf life, but this is long after most of the chlorophyll has been degraded. This increase in ABA levels at time of leaf death has been shown to be almost universal across land plants (McAdam, Kane, Mercado Reyes, Cardoso & Brodribb 2022). An area that requires further investigation is the effect of closed stomata on leaf senescence independent of ABA. It has been proposed that ABA may drive leaf senescence indirectly by closing stomata and reducing photosynthesis (Thimann & Satler 1979b a; Wagner, DeFoliart, Doak & Schneiderheinze 2008). The work Thimann and Satler is based on sections of oat leaves exposed to various substances that can either force stomata open or closed. I plan to investigate whether stomatal closure, independent of ABA, can induce leaf senescence. I would propose to do this by artificially blocking the stomatal surfaces on leaves of deciduous trees with petroleum jelly in addition shading branches of sun

adapted branches to close stomata while tracking chlorophyll content and photosynthesis. This aligns with previous research indicating that ethylene, the hormone primarily associated with leaf senescence, can also play a role in maintaining stomatal closure (Bi *et al.* 2023). One possible mechanism for this could be the fact that ethylene is a gas and if stomata are closed it may slow the escape of the gaseous ethylene leading it to build up in the leaf enhancing senescence.

There are several areas related to the end of leaf life that I intend to explore further, with a particular focus on the process of abscission. Abscission typically follows senescence, wherein leaves are actively shed from the plant. This process is believed to be closely associated with the movement of auxin out of leaves through the petiole and across the abscission zone, an area of compressed cells located at the junction of the petiole and branch (Morris & Small 1990; Dong *et al.* 2021; Moretti & Souza 2023). Once auxin ceases to move through the abscission zone, cells undergo programmed cell death until the leaf is only connected via the already dead xylem (Wetmore & Jacobs 1953; Morris & Small 1990; Dong *et al.* 2021). I am hypothesizing that this process may be influenced by photosynthesis, considering some findings that suggest a correlation between auxin levels in mature leaves and their exposure to light (Osborne & Hallaway 1964).

Another area deserving deeper investigation is that of leaf marcescence, a phenomenon observed in temperate trees where dead leaves are retained throughout the winter and only shed during the spring, coinciding with the emergence of new leaves. Marcescence has been attributed to various causes and effects (Heberling & Muzika 2023). One hypothesis proposes that the dry leaves reduce deer browsing by creating noise when brushed against (Svendsen 2001). Another suggestion is that the shedding of dry leaves in spring facilitates decomposition, allowing the reintroduction of nutrients just when the tree needs them most for spring leaf out (Angst *et al.* 2017). Notably, significant anatomical research on marcescent leaf abscission zones was conducted at Purdue in 1949, focusing on two species of *Quercus*. The study revealed that these species lack a well-defined abscission zone at the base of the leaves; even the petioles seem to have living tissues at their base, while the blade and the majority of the petiole are dead. Tissue death occurs only in late winter and spring at the petiole's base, requiring mechanical separation, such as wind, for the leaf to finally detach (Hoshaw & Guard 1949). I am intrigued by the possibility that holding onto leaves longer into autumn may extend the growing season slightly or allow for a more complex extraction of mobile nutrients from the leaves. This aligns with the observation that some marcescent species tend to become more deciduous once they reach maturity

(Svendsen 2001; Heberling & Muzika 2023). If marcescent leaves indeed extend the growing season, a critical period for this could be when trees are first growing and competing against neighboring saplings.

## 7.4 Freezing

If trees retain their leaves in the autumn, these leaves must withstand repeated freezing events. Frost events, both at latitude and altitude, play a crucial role in determining species distribution (Tranquillini 1982; Inouye 2000; Walker *et al.* 2004; Stuart, Choat, Martin, Holbrook & Ball 2007; Löffler 2007). Brevideciduous species exhibit the ability to survive and tolerate repeated sub-zero temperatures while maintaining photosynthesis during warmer periods (Taneda & Tatenno 2005; Koehler, Center & Cavender-Bares 2012). Even evergreen species, though capable of surviving freezing events, have limits beyond which leaf function becomes unrecoverable (Ashworth 1993).

In Chapter 5, I developed a novel method for visualizing freezing initiation in leaves of *Lonicera x purpusii* using time-lapse photography. This technique, employing Raspberry Pi cameras, allows for a cost-effective and relatively simple assessment of freezing temperature and ice spread in different leaf tissues, akin to tracking embolism in drying leaves (Cardoso, Kane, Rimer & McAdam 2022). This method may facilitate easier phenotyping of plants for frost resistance and tissue freezing points. Furthermore, my experiments revealed that freezing point tests must closely mimic natural temperature declines, as too rapid freezing can artificially alter the freezing point. I also observed that leaves of *L. x purpusii* can freeze and thaw multiple times while still recovering photosynthesis. Additionally, I determined the minimum temperature at which leaves of *L. x purpusii* can tolerate damage to be -19°C.

To advance research in cold temperature plant biology, I am interested in exploring the possibility that brevideciduous shrubs, such as *L. x purpusii*, might exhibit low levels of photosynthesis when leaf temperatures are below 0°C but above the freezing point of ~-4.5°C. Observations in alpine herbs and phytoplankton suggest that photosynthesis can occur at sub-zero temperatures. Considering the color and surface area of leaves, they may thaw earlier than branches, potentially leading to water transpiration through the cuticle and partially closed stomata. This could result in leaves experiencing drought-like conditions until the branch xylem unfreezes. Moreover, I would like to investigate the dynamics of freezing recovery in deciduous trees. While

frost is believed to cause embolism in woody organs, and embolism is typically considered unrecoverable, these trees can restore conductivity to burst buds in the spring (Sperry, Nichols, Sullivan & Eastlack 1994; Langan, Ewers & Davis 1997; Utsumi, Sano, Funada, Fujikawa & Ohtani 1999; Mayr, Gruber & Bauer 2003; Koehler *et al.* 2012). This recovery might occur through the expulsion of air from the xylem using high root pressure, or the old embolized xylem might not be recovered, being replaced annually with entirely new xylem (Cochard, Lemoine, Améglio & Granier 2001; Lobos-Catalán & Jiménez-Castillo 2023).

## **7.5 Tree phenology in a changing world**

It has recently been proposed that deciduous tree senescence is triggered by the filling of carbon sinks (Zani, Crowther, Mo, Renner & Zohner 2020, 2021; Zohner *et al.* 2023). According to this theory, once trees complete their seasonal photosynthesis and fill their carbon sinks, they undergo senescence and shed leaves. Consequently, if warmer springs due to climate change lead to earlier leaf out, it could result in early autumn senescence due to additional carbon gain during the early season. However, this theory has sparked controversy, as some studies have found no clear link between early leaf out and early autumn senescence (Norby 2021; Lu & Keenan 2022). Observations, including those from the Free Atmospheric CO<sub>2</sub> Experiments (FACE), contradict this proposed mechanism (Norby 2021).

To explore the impact of warmer temperatures on growing seasons and senescence, I conducted a comparative study during a Fulbright Fellowship in Tasmania, Australia, the results of which are briefly outlined in Chapter 6. Contrary to the initial theory, the study found that most trees in the warmer spring conditions of Tasmania extended their growing seasons rather than shortening them. Additionally, there was a considerable diversity in responses to warming, with some species extending their growing seasons in both spring and autumn, some experiencing early leaf out, and others showing strong photoperiodism, with no observable effect on growing season length due to changes in location and temperature.

To deepen our understanding of source-sink relationships in long-lived species, further investigation is essential. Some studies suggest that summer droughts, which reduce photosynthesis, can delay autumn senescence, indicating that the inability to fill carbon sinks may lead to postponed senescence. I aim to explore how drought may influence the phenological dynamics of temperate deciduous forests. Additionally, manipulating source-sink relationships

by reducing leaf area and observing the impact on end-of-season dynamics is an area that requires further exploration.

## 7.6 Conclusions

In this thesis, I delved into various research areas spanning the entire lifecycle of leaves. I uncovered that expanding leaves lose a significant amount of water, with a majority of it being lost through cuticles rather than open stomata. I also identified ABA as a crucial driver of stomatal closure during drought in an anisohydric species. While ABA can enhance autumn leaf senescence, it is not typically produced during that time. Additionally, I developed a novel method for observing leaf freezing events and found substantial diversity in phenological responses to a warming climate across different species.

Trees remain a challenging subject of study due to their long lifespan and slow lifecycle. To fill these gaps in our understanding, assumptions about deciduous trees have often been based on work done in model species like *Populus* or, even more problematically, in herbs and annuals. Through this research, I've come to realize that at every stage of leaf life, there exists a remarkable diversity among plant life. This diversity extends even within deciduous trees, emphasizing the importance of avoiding assumptions based on observations made in single species, which may not even be deciduous trees. Instead, addressing questions with a diverse range of species that inhabit the target environment provides more meaningful insights.

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