THESIS

LONG TERM *IN VIVO* OBSERVATION OF MAIZE LEAF XYLEM EMBOLISM, TRANSPIRATION, AND PHOTOSYNTHESIS DURING DROUGHT AND RECOVERY

Submitted by

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In partial fulfillment of the requirements

For the Degree of Master of Science

Colorado State University

Fort Collins, Colorado

Fall 2024

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ABSTRACT

LONG TERM *IN VIVO* OBSERVATION OF MAIZE LEAF XYLEM EMBOLISM, TRANSPIRATION, AND PHOTOSYNTHESIS DURING DROUGHT AND RECOVERY

Plant water transport is essential to maintain turgor, photosynthesis, and growth. Water is transported in a metastable state under large negative pressures, which can result in embolism, i.e., the loss of function by the replacement of liquid xylem sap with gas, as a consequence of water stress. Unfortunately, the detection of embolism is difficult because any manipulation of the xylem to facilitate measurement (e.g., cutting) can unintentionally introduce embolism. Therefore, our understanding of the timing of embolism, relative to other physiological responses, is incomplete. To avoid experimental artifacts, we used non-invasive methods to quantify embolism occurrence in maize leaves to characterize the sequence of physiological responses (leaf shrinkage, photosynthesis, chlorophyll fluorescence, and transpiration) during severe water stress. Embolism formation occurred after other physiological processes decreased and was irreversible upon rewatering. Recovery of transpiration, net CO2 assimilation, and photosystem II efficiency were aligned with the severity of embolism, whereas these traits returned to near pre-stress levels in the absence of embolism. A better understanding of the relationships between embolism occurrence and downstream physiological processes during stress and recovery is critical for the improvement of crop productivity and resilience.

ACKNOWLEDGMENTS

First and foremost, I would like to thank my committee members, Dr. Sean Gleason, Dr. Troy Ocheltree, and Dr. Alan Knapp for their continued support. Their mentorship and feedback have been invaluable in shaping who I have become, both personally and professionally. I am forever grateful to Sean for his guidance and support in shaping my career. I would also like to thank Troy for his support in both the classroom and the lab. I would also like to thank the accountabilibuddies group for helping me keep track of my goals and holding me accountable. I want to express my gratitude to Dr. Cameron Hunter for introducing me to research and the exciting world of plant physiology. I want to thank Dr. Jared Stewart for his patience, wisdom, and encouragement. I want to thank Dr. Stephanie Polutchko for getting me across the finish line and for being one of my very best friends. I'm also grateful to the other accountabilibuddies both inside the Ocheltree lab and at the Water Management and Systems Research Unit, who have supported me throughout my professional development, including Dan Spitzer, Naiara Doherty Garcia, David Barnard, Kendall DeJonge, Adam Mahood, Rob Erskine, Huihui Zhang, Louise Comas, Kyle Mankin, and Kevin Yemoto for cheering me on every step of the way and helping me drink substantial amounts of coffee.

I would also like to thank my family Kelly, James, and Camryn and friends for supporting me as I continued through school. I would like to extend a special thank you to Lauryn, cats Bean and Ivy, as well as shrimp and catfish, for supporting me through the late nights working on this thesis. Finally, I would like to thank several hundred corn plants for their contribution and service to science.

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CHAPTER 1: INTRODUCTION

Herbaceous, non-woody, plants are composed predominantly of water, which typically accounts for 80–95% of their fresh mass. Thus, the efficient transport of water from the soil to where it is needed is critical for maintaining plant turgor, photosynthesis, growth, and producing harvestable products (i.e. grain, forage, biomass). When water is withheld the cessation of growth and subsequent wilting are usually the first signs of stress noticed by concerned observers, but alone are poor predictors of irreversible damage to the plant. These symptoms are often reversed soon after water becomes available again. Still, when these symptoms persist in a limited capacity or worsen, they might indicate dysfunction within the water transporting system of plants, the xylem.

As a consequence of drought stress, the tension within the xylem can increase to a critical point at which air can enter xylem conduits and create blockages by replacing the liquid xylem sap with water vapor, resulting in embolism, which impedes water transport to plant tissues downstream of the embolism (Zimmermann, 1983). The tension within the xylem presents difficulties when trying to quantify lethal levels of drought stress as most traditional methods rely on some manipulation of this system (i.e., cutting under tension), which may accidentally introduce embolism and prevent the direct observations of the physiological responses to accumulated embolism on recovering plants, or give the appearance of sudden overnight refilling when plants are cut again under less tension (Torres-Ruiz et al., 2015).

1.1. Methods development

The difficulties associated with observing the direct consequences of xylem embolism prompted the development of new non-destructive methods used in the following study. Based on the optical vulnerability technique introduced in 2016 (Brodribb et al., 2016), a custom-built time-lapse imaging apparatus, hereafter death cam, would be placed on the six most recent fully expanded leaf tips of maize. The death cams work on the same principle outlined by Brodribb et al. (2016), where a sequence of images can reveal the timing and location of leaf vein embolism.

The death cam works by positioning the leaf in a fixed position for continuous imaging of the adaxial leaf surface. When the xylem vessels in leaf veins embolize the replacement of liquid sap with gas is apparent by a subtle change in light refraction within the vein which appears as a slight darkening of the leaf vein when viewed in a sequence of images. In the clamp, the adaxial side of the leaf is placed flat against a standard slide where it is imaged by the USB camera in the housing below. The camera's lens was unscrewed approximately 7mm, magnifying a section of the leaf (c.a. 16 cm^2). Stacked above the leaf, a photo-resin printed diffuser plate holds the leaf in a fixed position by securing the midrib of the leaf in the corresponding channel. Situated above the diffuser plate is the light housing where, a generic white 5W LED chip (powered at 5V, with a 150-ohm resistor) provides transmission illumination through the leaf. Utilizing an Arduino-based switchboard, the housing light can be illuminated only during imaging. The six cameras were multiplexed to a Unix computer where a python script using the open-cv library would capture images at a specified interval and turn on and off the associated lights for each camera housing.

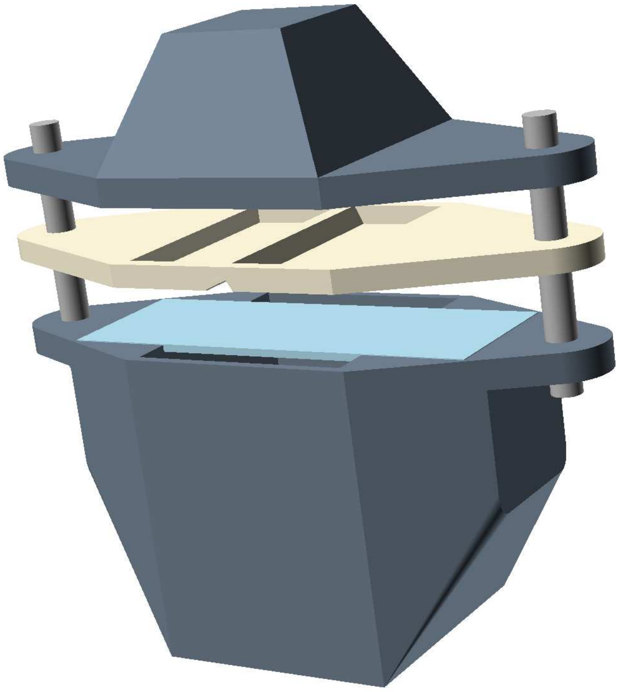

Figure 1.1. Death cam rendering. Camera base (bottom) containing Arducam 2MP OV2710 USB camera (not pictured), standard microscope slide. Diffuser plate (middle) with midrib channel, and light housing (top) containing white led chip (not pictured). Two 3/16" bolts hold the housing components together with wing nuts loosely tightened to secure the adaxial leaf surface flat to the microscope slide.

In addition to leaf embolism the leaf images were also used to quantify leaf shrinkage represented here as inter-vein distance. Leaf veins on either side of the midrib are thresholded and the pixel position of each vein is recorded using the 'center of mass' selection from the 'Analyze Particles' function in Fiji (which is just ImageJ; Schneider et al., 2012). Leaf shrinkage is a useful proxy for the internal water status of the leaf (Scoffoni et al., 2014). Because maize and other grass species follow a conserved parallel venation structure there is more resolution in lateral shrinkage than in leaf thickness which is more applicable to dicotyledenous species with reticulate venation. Leaf shrinkage adds a more nuanced look into the regulation of leaf water after stomatal closure where gas exchange measurements begin to lose utility. Additionally, leaf shrinkage may be useful in evaluating the contribution of tissue capacitance to leaf longevity under acute drought events.

The following set of experiments focused on assessing the formation of embolism and the subsequent physiological consequences of embolism on leaf-level physiological traits. By placing the newly developed optical sensors on the six uppermost developed leaves of a vegetative maize plant, a spatial and temporal gradient of embolism onset was observed. Additionally, gas exchange system sensor heads were placed directly adjacent to the death cams on leaves 8,10, and 13, pairing embolism and leaf shrinkage measurements to an incredible gas exchange dataset. The depressed recovery of photosynthesis, stomatal conductance, and photosynthetic efficiency was linked to accumulated embolism. This addressed a critical question of how embolism influenced the recovery of leaf function following a severe embolism-inducing drought event.

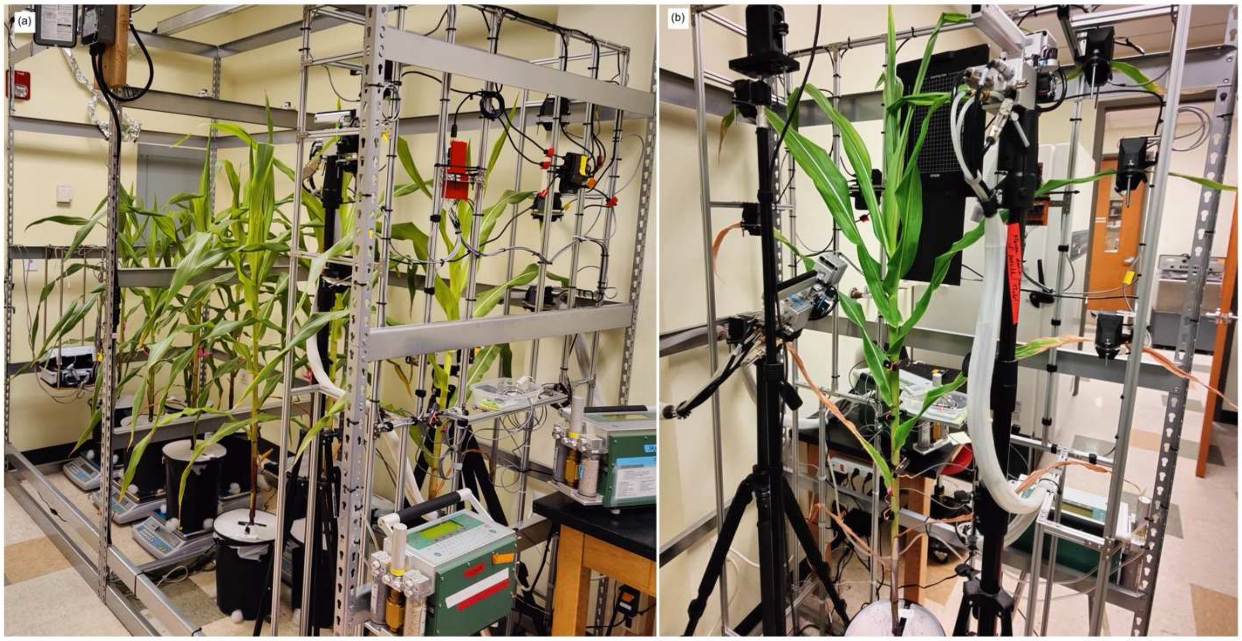

Figure 1.2 Images of the experimental setup, including companion plants for destructive harvest (a). The instrumented plant with accompanying optical sensors on leaves 8-13, and LI-6400/XT placed on leaves 8 and 13 (b).

CHAPTER 2: LONG TERM *IN VIVO* OBSERVATION OF MAIZE LEAF XYLEM EMBOLISM, TRANSPIRATION, AND PHOTOSYNTHESIS DURING DROUGHT AND RECOVERY[1]

1. Introduction

Water movement within plants is essential for critical functions, including CO_2 assimilation, growth, and turgor. Water moves from the roots to the sites of photosynthesis through xylem conduits under negative pressure. The high tension inherent in these conduits can result in embolism formation, which are gas blockages disrupting the transport of liquid water (Sperry and Tyree, 1988; Tyree et al., 1986). Embolism formation introduces hydraulic resistances within the xylem system, which impacts leaf-level water utilization and recovery after re-watering (Gleason et al., 2017b; Nadal et al., 2023). The interconnectivity of traits influencing water transport and utilization forms a finely tuned network shaped by both natural and artificial selection (Brodribb and Holbrook, 2003; Carlquist, 2012; Cochard, 2002; Gleason, 2015). Understanding the relationships between embolism and leaf physiology is critical for ensuring resilient and productive agricultural practices in the face of evolving environmental challenges.

While claims suggest that maize, among other species, can experience embolism formation and subsequent refilling overnight (Gleason et al., 2017a; McCully, 1999; Tyree et al., 1986), we contend that artifacts associated with the destructive/invasive methods used in these experiments (e.g., “long vessel”, “cutting” artifacts), have likely resulted in the overestimation of conductivity loss and recovery (Cochard and Delzon, 2013; Torres-Ruiz et al., 2015; Wheeler et al., 2013). This

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[1] This work was submitted to *Plant, Cell & Environment* for review in August 2024 as: Allen BS, Stewart JJ, Polutchko SK, Ocheltree TW, Geason SM. Long-term *in vivo* observation of maize leaf xylem embolism, transpiration, and photosynthesis during drought and recovery.

discrepancy prompted our development and use of non-destructive methodology focused on identifying water potentials associated with key physiological thresholds in this important species, such as stomatal closure, decline in photosystem II (PSII) efficiency, embolism, and embolism reversal (if observed). Additionally, we sought to understand the relative timing of these thresholds during prolonged drought, as well as the timing of their recovery after plants were returned to a fully hydrated state.

Understanding the sequence of these thresholds during drought, as well as their associated water potentials, is critical because the vast majority of crop physiological research has focused on improvement of stomatal and photosynthetic functioning and/or root traits (timing, magnitude, response to soil and climate cues; Ainsworth and Ort, 2010; Lopes et al., 2011; Palta and Turner, 2019; White, 2019), without consideration for the integrity of the liquid water transport pathway, which is vulnerable during drought (Brodribb et al., 2007; Gleason, 2015). It is, therefore, critical that we know the *relative* importance of these thresholds, the connections between them, as well as their recovery (or lack of) after drought so crop improvement programs can properly prioritize the most likely trait networks that confer improved performance (Gleason et al., 2022). Leaves, being the primary sites of evaporation, and susceptible to variation in light and evaporative demand, are often the first organs to experience detrimental effects due to embolism (Song et al., 2022). By investigating the implications of embolism accumulation, we aim to shed light on the intricate dynamics of leaf response to water stress.

Leaf shrinkage has garnered attention for its potential implications in drought stress responses (Canny et al., 2012; Johnson et al., 2018; Scoffoni et al., 2014). Water released from shrinking tissues can be quantitatively linked to the delay of embolism and leaf death after stomatal closure (Blackman et al., 2016; Gleason et al., 2014). Connecting leaf shrinkage, an easily measurable trait, with physiological processes could allow for easier identification of critical thresholds during drought periods, especially when other measures of plant function (e.g., net CO_2 assimilation and transpiration) have already reached depressed steady states lacking informative resolution (Bourbia et al., 2022).

This study aims to identify when embolism occurs when leaves are subjected to low water potential, particularly in relation to the decline of essential leaf functions and physiological processes. We conducted this investigation in laboratory conditions across canopy leaves during a controlled dry down of potted maize plants. We focus on four key physiological traits -- leaf embolism, whole-plant transpiration, net CO_2 assimilation, PSII efficiency, and leaf shrinkage -- and ask four questions concerning the timing and functioning of these traits: 1) at what water potential does leaf embolism occur in maize leaves and how does the timing and associated water potential differ across all leaves in the canopy; 2) what water potential does stomatal conductance and PSII efficiency become meaningfully reduced/impaired during drought, and how well are these declines aligned with embolism; 3) does stomatal conductance and PSII efficiency recover after drought, and if so, is the timing and/or magnitude of this recovery associated with the amount of embolism; and 4) can the loss of water volume from leaves during drought (hereafter "shrinkage") be used as a proxy trait to predict the decline and/or recovery of these physiological traits?

2. Materials and methods

2.1. Plant material and growth conditions

Zea mays L. subsp. *mays* genotype B73 was grown from seed (obtained from USDA-ARS Germplasm Resources Information Network; accession PI 550473) under greenhouse conditions (see below) at the USDA Crops Research Laboratory, Fort Collins, CO, USA. The use of B73 was deliberate due to its fully sequenced genome, aligning with our broader objective of identifying traits that support crop resilience by establishing meaningful benchmarks for plant stress in this model genotype. A drip-line irrigation system, regulated by a battery-operated timer (RBC7000, DIG, Corp., Vista, CA, USA) connected to 1.9-cm diameter poly tubing, delivered water to the plants at regular 12-hour intervals. Plants were grown in 15 L nursery pots filled with Profile Greens Grade (PROFILE Products LLC, Buffalo Grove, IL), a non-nutritive calcined clay substrate with favorable water retention properties. Nutrients were provided by 15 g of Osmocote Plus slow-release fertilizer (The Scotts Miracle-Gro Company; Marysville, OH, USA), which was mixed homogeneously into the substrate.

After approximately seven weeks of growth (i.e., 50 days after seeds were sown), plants were moved to laboratory conditions and placed in a custom-built growth cage (Figure 2.1, see also Gleason et al., 2024) with a 15-hour photoperiod (05:30 to 20:30) provided by two rows of Philips GreenPower LED top lighting (maximal light intensity of 600 μmol photons m^{-2} s^{-1}). The relative humidity was 19.2 ± 11.9%, temperature was 24.5 ± 0.6°C, and vapor pressure deficit (VPD) was 2.49 ± 0.39 kPa. Once plants were in the lab space, water was withheld until xylem embolism was observed in the majority of upper leaves (typically ca. 20–50%). This range in leaf xylem embolism was intentional, as we aimed to examine the effect of embolism on the speed and completeness of recovery after plants were re-watered. At the time of rewatering, approximately 3 L of water was added to each pot to saturate the substrate. Plants remained well watered for the duration of the six day recovery period.

2.2. Water potential

Plants were grown in four cohorts of eight plants, with one plant from each cohort being selected for instrumentation and non-destructive measurements (Figure 2.1; see below). The other plants were subjected to periodic water potential measurements using a Scholander-type pressure chamber (1505-D, PMS Instruments, Albany, OR, USA). For some plants, water potential was measured from every expanded leaf available at a single time point. This spatial variability made it difficult to assign a single water potential to the whole plant because leaves were found to become hydraulically isolated from the rest of the plant as they desiccated completely (Figure 3.1).

2.3. Leaf optical measurements

Embolism formation and change in inter-veinal distance were assessed with custom-built, open-source optical sensors described by Gleason et al. (2024) based on methods described by Brodribb et al. (2016). Six sensors were positioned approximately 15 to 30 cm from the tip of the six most recently collared leaves (i.e., leaves 8 to 13 from the bottom of the plant; Figure 2.1). Each optical sensor imaged a leaf area of approximately 16 cm^2. The adaxial side of the leaf was loosely held flat against a microscope slide at the imaging plane. A 3D-printed clear resin diffuser plate, with a channel for the midrib, was positioned between the LED light and the leaf, stabilizing the leaf during experimentation but still allowing for leaf shrinkage. Optical sensors were secured with aluminum T-slot framing clamped to the camera housing. Generic 5W white LEDs were placed in the center of the light housing to provide light only during the imaging process. USB cameras, 2MP OV2710 (Arducam Technology Co., Kowloon, Hong Kong, PRC) were used to image the adaxial face of the leaf. Images were taken in 5-minute intervals using OpenCV-Python controlled by a UNIX computer (Ubuntu 22.04 LTS).

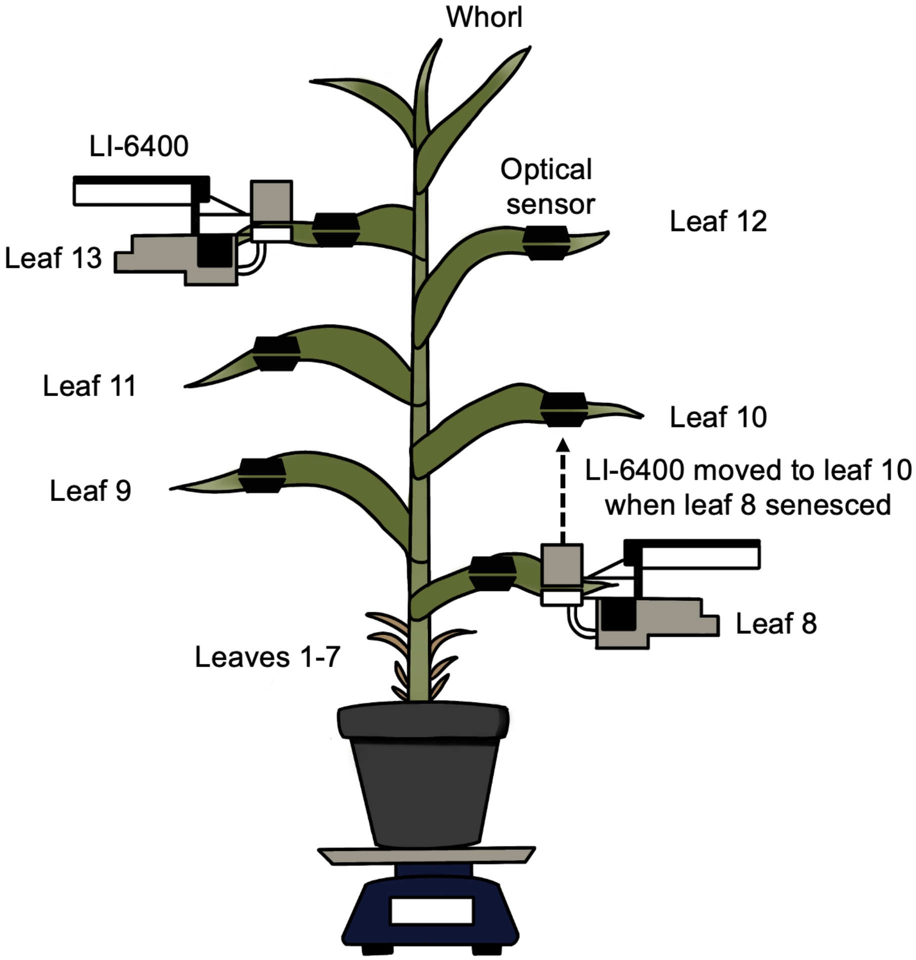

Figure 2.1. Schematic diagram of experimental setup.

The image stacks generated by the optical sensors were evaluated for leaf vein embolization using ImageJ software (Schneider et al., 2012). Leaf shrinkage was calculated by taking the difference in pixel positions and expressing values as a percent change from the initial distance between leaf veins (Patil et al., 2022). Embolism was expressed as a ratio of embolized leaf veins to the total number of visible leaf veins in the optical sensors, and expressed as a percentage. This approach was adopted to ensure accuracy in our assessment of embolism, as alternative methods of analyzing optical embolism data, such as assigning a uniform value to embolism events regardless of size, may not accurately reflect declines in hydraulic conductance (Cardoso et al., 2020; Venturas et al., 2019).

2.4. Gas exchange and chlorophyll fluorescence

Two LI-6400/XT Portable Photosynthesis Systems (LI-COR Biosciences, Lincoln, Nebraska, USA) with 6400-40 fluorometer chambers were attached to leaves 13 and 8 and were later moved to leaf 10 after leaf 8 senesced (Figure 2.1). Measurements of gas exchange as well as maximal (F_m') and minimal (F_o') yields of chlorophyll fluorescence were taken at 15-minute intervals from the same leaf position using the ‘Auto Log 2’ function with the chamber set to replicate the prevailing light intensity. Photosynthetic efficiency was calculated as $(F_m' - F_o')/F_m'$ (Schreiber et al. 1986). Plant transpiration was calculated from changes in mass, which were measured using digital balances (Adam CBK 70A, Adam Equipment, Inc., Oxford, CT, USA) and logged every 30 seconds. To minimize changes in mass due to substrate evaporation, the tops of pots were wrapped with white plastic. Transpiration measurements were obtained from the instrumented plant and up to six accompanying plants.

2.5. Statistical analysis

Leaf water potential was estimated from individual leaf water potential measurements taken from companion plants and predicted using the time after stomatal closure with linear least squares regression with the “lm” function in R (R Core Team, 2021; Figure 3.1). Increasing embolism and decreasing gas exchange and chlorophyll fluorescence parameters with declining water potential were fit with local polynomial regression models using the “loess” function in R. In each case, 12%, 50%, and 88% declines in leaf function were determined, and standard deviations were calculated across the four cohorts using cohort mean values (Table 1).

Relationships between embolism accumulation and recovery of photosynthesis after re-watering were evaluated with ordinary least squares regression using the "lm" function in R.

Table 1. Water potentials (MPa) corresponding to 12% (P_{12}), 50% (P_{50}), and 88% (P_{88}) of xylem embolism in leaves 8–13, loss of CO_2 assimilation, loss of stomatal conductance, and loss of photosynthetic efficiency in leaves 8 & 13.

	P_{12}	**P_{50}**	**P_{88}**
Embolism (leaf 13)	-1.76*	-2.28*	-2.37*
Embolism (leaf 12)	-1.79 (-1.60, -1.98)†	-2.16 (-1.91, -2.41)†	-2.33 (-2.18, -2.48)†
Embolism (leaf 11)	-1.85*	-2.48*	-2.74*
Embolism (leaf 10)	-1.70 (-1.55, -1.84)†	-2.13 (-2.00, -2.26)†	-2.56 (-2.52, -2.61)†
Embolism (leaf 9)	-1.42 ± 0.09	-1.65 ± 0.20	-1.87 ± 0.20
Embolism (leaf 8)	-1.25 ± 0.22	-1.34 ± 0.22	-1.52 ± 0.15
CO_2 assimilation (leaf 13)	-1.13 ± 0.04	-1.23 ± 0.03	-1.44 ± 0.06
CO_2 assimilation (leaf 8)	-1.12 ± 0.08	-1.2 ± 0.09	-1.31 ± 0.09
Stomatal conductance (leaf 13)	-1.13 ± 0.04	-1.23 ± 0.03	-1.44 ± 0.06
Stomatal conductance (leaf 8)	-1.12 ± 0.08	-1.2 ± 0.09	-1.31 ± 0.09
Photosynthetic efficiency (leaf 13)	-1.14 ± 0.03	-1.29 ± 0.03	-2.07 ± 0.19
Photosynthetic efficiency (leaf 8)	-1.16 ± 0.08	-1.26 ± 0.1	-1.41 ± 0.08

n = 4 mean and standard deviation unless indicated; * *n* = 1 calculated value; † *n* = 2 mean and range.

3. Results

3.1. Onset of leaf xylem embolism during dry down

Initial embolization events in the upper leaves of maize started ca. day 15 (Figure 2.2) when water potential was ca. −1.7 MPa (Table 1). In cases where water was withheld longer than 20 days, all upper leaves were embolized before day 30 (Figure 2.2a,b,c). After day ten of the dry down, leaf 8 had begun to embolize and progressed to 100% before day 20 (Figure 2.2f). Leaf 9 was fully embolized within 15 days (Figure 2.2e). In contrast, the upper leaves (leaf > 11) did not continue to embolize after plants were re-watered (Figure 2.2d). Leaves 11 through 13 behaved similarly after the onset of embolism, exhibiting a gradual accumulation of embolism, in contrast to leaves 8 and 9, which desiccated much more rapidly and were fully embolized earlier in the dry down. Given these differences in leaf senescence, there was notable variability in the timing and degree of embolism across leaves and replicates. Little embolism was observed in the upper leaves, while leaf 8 and leaf 9 were embolized (Figure 2.2e,f) and senesced within the first ten days of the dry down. The first three cohorts were re-watered when embolism was observed in some veins of the upper leaves (approximately 20 days into the dry down), whereas the final cohort was dried until embolism was observed in all leaf veins in all upper leaves (i.e., plants were not re-watered). This contrast between upper and lower leaves is further reflected by the leaf water potential corresponding to initial embolization events, (P_{12}), predicted to be $\Psi_{leaf} < -1.7$ MPa for leaf 13, whereas in lower leaves, 8 and 9, P_{12} was predicted to be $\Psi_{leaf} > -1.5$ MPa (Table 1).

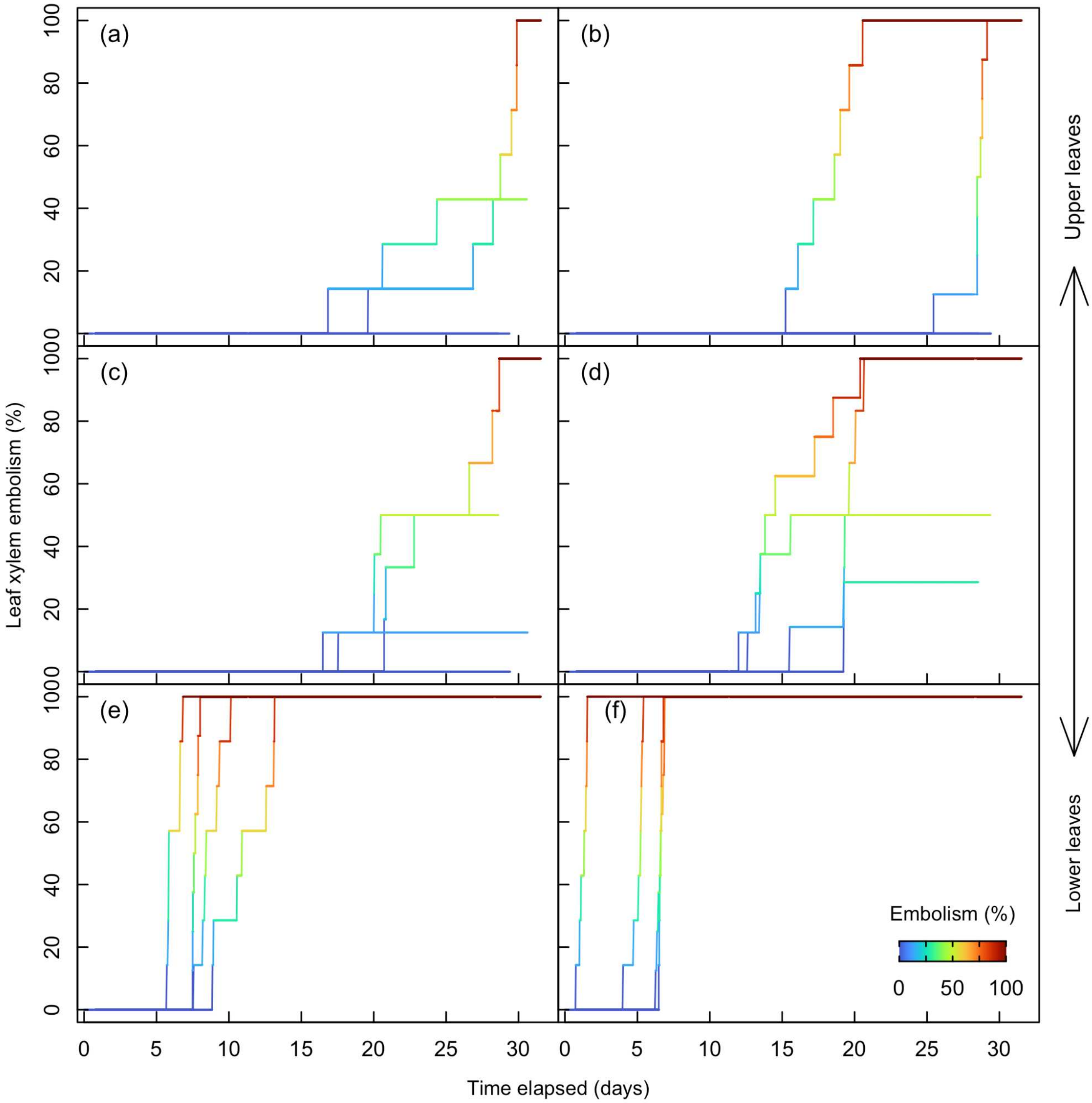

Figure 2.2. The relationship between xylem embolism in the 13th (a), 12th (b), 11th (c), 10th (d), 9th (e), and 8th (f) leaves and time elapsed. Symbols are colored by the percentage of leaf vein bundles with embolism within each leaf. *n* = 4 plants.

3.2. Decline of transpiration and photosynthesis during dry down

Transpiration was sustained at maximal levels before declining around day 5 and incrementally decreasing each subsequent day (Figure 2.3a). Net CO_2 assimilation in the upper leaves (leaf 13) declined from maximum values within five days of dry down (Figure 2.3b) when water potential was ca. −1.1 MPa (Table 1). Matched by declines in daytime photosystem II (PSII) efficiency and eventually reaching near-zero levels after day 20 (Figure 2.3d), at which point water potential was ca. −2 MPa (Table 1). In the lower leaves (leaves 8 & 10), net CO_2 assimilation, stomatal conductance, and photosystem II efficiency declined in concert to a minimal level before day 5 (Figure 2.3c) at a water potential of ca. −1.3 MPa (Table 1). The senescence of leaf 8 is noted by the sharp decline in PSII efficiency to 0 at roughly five days into the dry down period (Figure 2.3e). At this point, the LI-6400/XT gas exchange system was moved from leaf 8 up to leaf 10, where assimilation and PSII efficiency had reached a steady, but depressed, state for the duration of the dry down until the desiccation of the leaf tissue within the cuvette, at roughly 20 days into the dry down (Figure 2.3d,e).

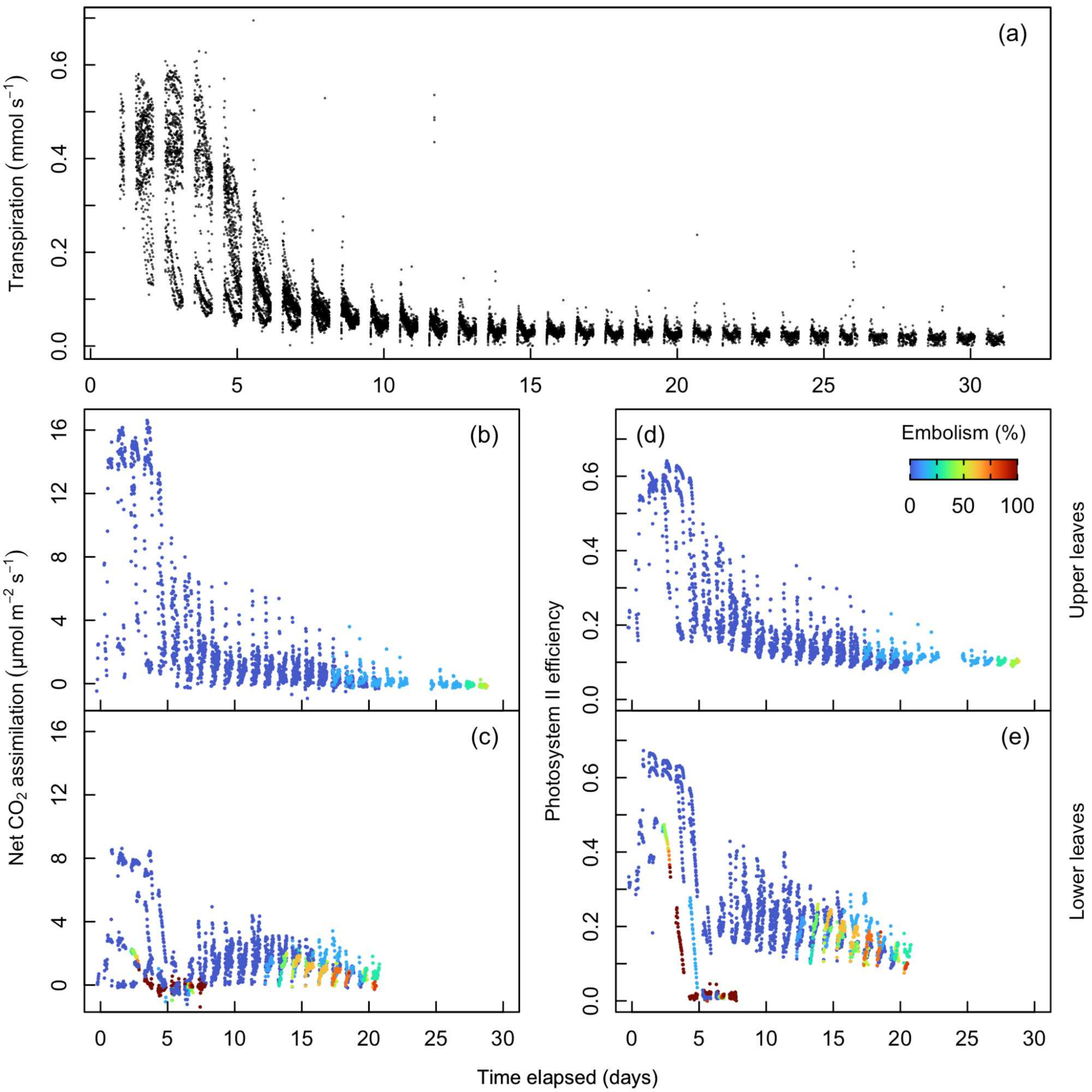

Figure 2.3. Whole-plant transpiration rate (a) and CO_2 assimilation rate (b,c) and photosynthetic efficiency (F_v'/F_m') (d,e) in upper (b,d) and lower (c,e) collared maize leaves during the dry down period. For (b–e), symbols are colored by the percent of major vein bundles with embolism within each leaf (see Fig. 1). *n* = 24 plants for (a) and *n* = 4 plants for (b–e).

3.3. Recovery of transpiration and photosynthesis

Transpiration was observed to recover to a steady state the day after water was added to pots, recovering between 52% and 107% (mean = 79.0%) of average pre-stress values (Figure 2.4a). Net CO_2 assimilation and PSII efficiency were measured for leaves 10 and 13. The upper leaf accrued up to a 43% reduction in the number of functional leaf veins during the dry down and recovered to a functional steady state two days after watering resumed. During recovery, CO_2 assimilation to 20% and 57% (mean = 43%) of pre-stress values was aligned with embolism severity ($R^2 = 0.93$; $P < 0.001$; Figure 4b). PSII efficiency recovered between 59% and 85% (mean = 75.6%) of pre-stress values, and similar to CO_2 assimilation, was also correlated with embolism severity ($R^2 = 0.88$; $P < 0.001$; Figure 4d). However, we note that because the gas exchange sensor head was moved to a higher leaf after leaf 8 senesced, these measurements were also taken under slightly higher light intensity (192 µmol photons m^{-2} s^{-1} on leaf 8 versus 218 µmol photons m^{-2} s^{-1} on leaf 10).

Of the plants that were re-watered, embolism was observed in 29% to 100% of vascular bundles in leaf 10, the lowest leaf that did not desiccate completely during the dry down. CO_2 assimilation and PSII efficiency returned to a steady state one day after plants were re-watered (Figure 2.4c,e). CO_2 assimilation and photosynthetic efficiency (Figure 2.4c,d) recovery trajectories were separated by the degree of embolism accumulated in the leaf, with differences being greater in the lower leaves (Figure 2.4c,e). Completely embolized leaves had little to no recovery of CO_2 assimilation and severely reduced recovery of PSII efficiency (Figure 2.4c,e).

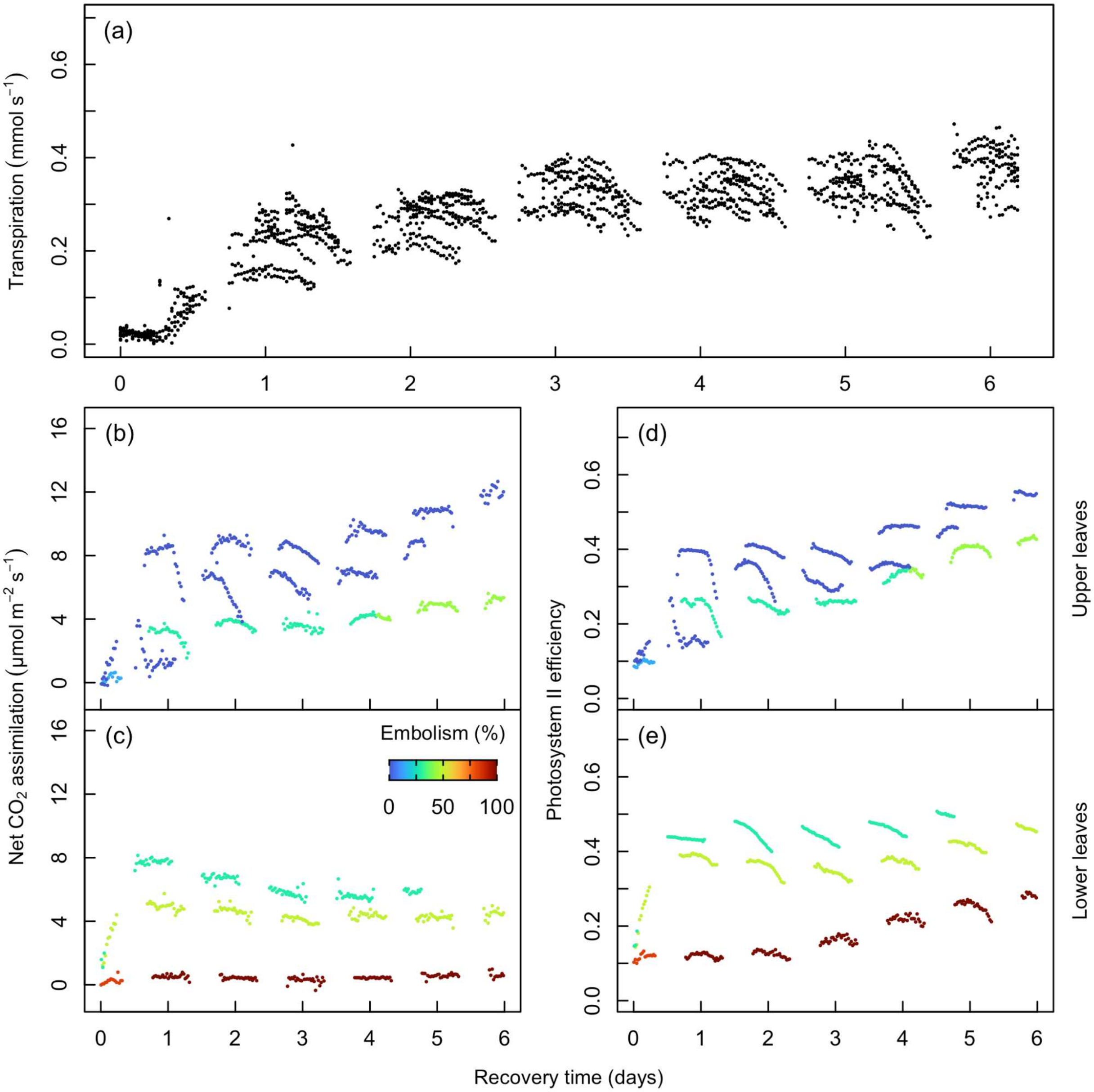

Figure 2.4. Whole-plant transpiration rate (a) as well as CO_2 assimilation rate (b,c) and photosystem II efficiency (F_v'/F_m') (d,e) in upper (leaf 13) (b,d) and lower (leaf 10) (c,e) leaves for six days after re-watering. Symbols are colored by the percent of major veins with embolism within each leaf (see Fig. 1). *n* = 12 plants for (a) and *n* = 3 plants for (b–e).

3.4. Shrinkage

In all leaves, the distance between veins on either side of the midrib decreased when water was withheld, and this was represented as a normalized fraction of the initial distance of leaf veins (Figure 2.5). Upper leaves experienced embolism much later into the dry down and were able to recover near initial values (Figure 2.5a). Most leaves that had experienced some embolism did respond to re-watering and recovered to near-maximal values within two nights of well-watered conditions (Figure 2.5b,c,d). In leaves where complete embolization of veins was observed, the inter-vein distance reached a minimal value and did not respond to re-watering (Figure 2.5d,e,f). Leaves 8 and 9 reached minimal values shortly after the complete embolization of the leaf's veins (Figure 2.5e,f). In most cases, the inter-veinal distance would decline by roughly 15–30% of initial values before localized leaf desiccation. In cases where minimal embolism was observed, and the loss of volume was less than 20%, leaves were able to recover to near initial inter-vein distance upon re-watering (Figure 2.5a,b,c,d).

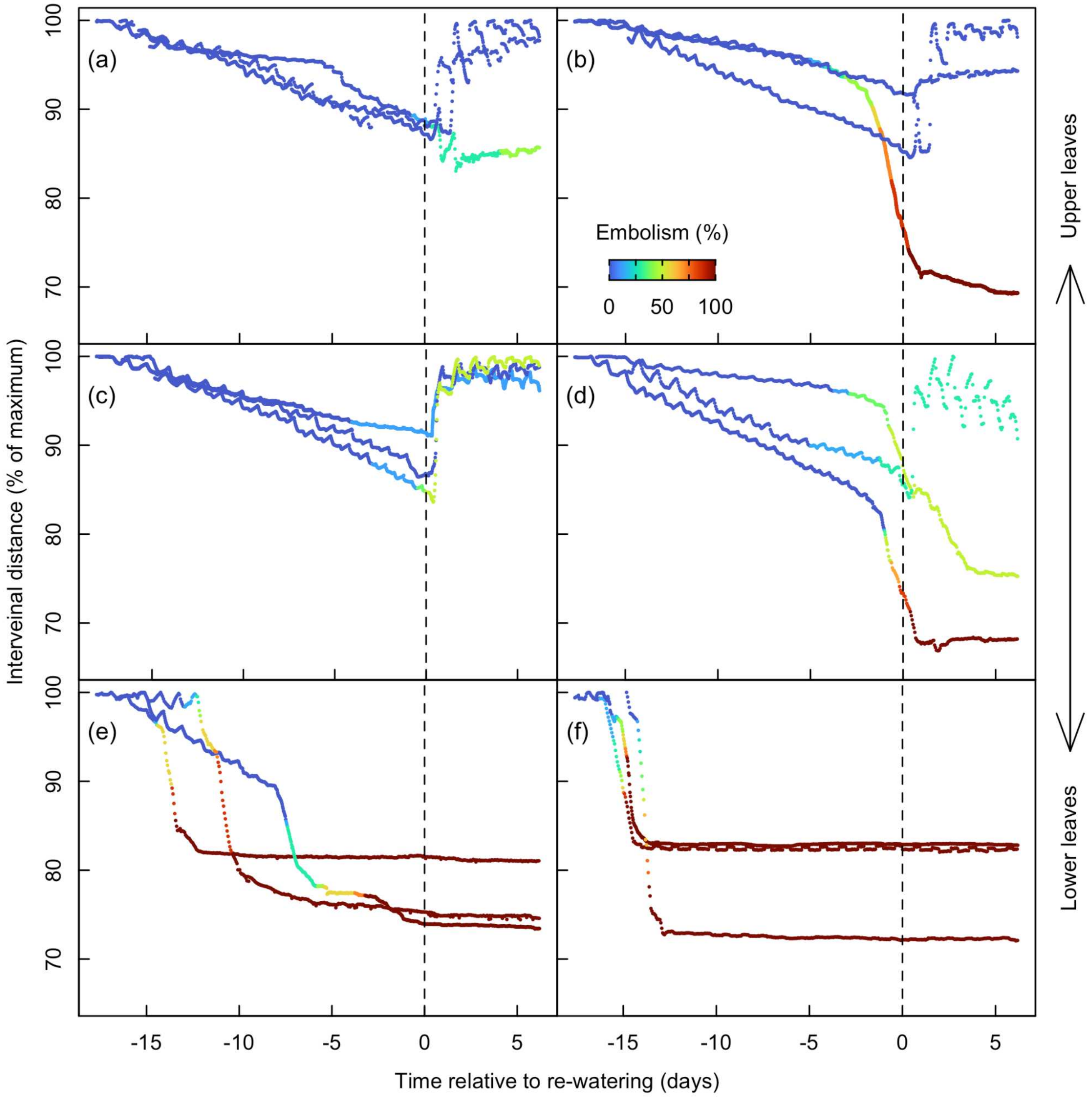

Figure 2.5. Inter-vein distance in the 13th (a), 12th (b), 11th (c), 10th (d), 9th (e), and 8th (f) collared leaves from the base. Vertical dashed lines indicate the time at which plants were re-watered (i.e., the end of the dry down period and the start of well-watered period). Symbols are colored by the percentage of leaf vein bundles with embolism within each leaf (see Fig. 1). *n* = 3 plants.

4. Discussion

This study evaluated embolism formation non-destructively during a dry down of intact maize plants and assessed the physiological consequences of accumulated leaf embolism across the canopy during recovery. We categorized several physiological leaf traits spatially and temporally in response to declining leaf water potential after withholding water and then subsequent recovery after re-watering. The sequence of decline in physiological traits during dry down was more or less aligned with our expectations, with the notable exception of a very long time period (ca. 15 days) between stomatal closure and embolism onset in upper (but not lower) leaves. During the dry down period, leaf shrinkage proceeded steadily and then greatly accelerated if/when embolism was observed. In both the upper leaves and lower leaves, the amount of accumulated embolism influenced the recovery of transpiration, photosynthesis, and PSII efficiency, highlighting the alignment among leaf shrinkage, embolism, and leaf physiological function during and following a prolonged drought.

4.1. Spatial and temporal patterns of stomatal closure and xylem embolism

In both the upper and lower leaves, initial declines in leaf-level physiological processes occurred at a similar time and water potential during the dry down (Brodribb and Holbrook, 2003; Gleason et al., 2017b). However, there was a pronounced time lag and slight pressure difference between the upper and lower leaves when large declines in photosynthesis and stomatal conductance were observed (i.e., P_{88}). The onset of embolism and subsequent tissue death occurred rapidly and earlier in the dry down period in lower leaves compared to upper leaves (Hochberg et al., 2017) because the lower leaves were more vulnerable (less negative P_{50}) to embolism formation than the upper leaves these results align with the segmentation hypothesis first proposed by Zimmerman (1983), and results in the preservation of higher leaf orders to benefit species during acute drought periods by reducing the maintenance and transpirational costs of less-productive leaves thereby lengthening the time to critical hydraulic failure in the upper leaves (Aparicio-Tejo and Boyer, 1983; Blackman et al., 2016; Gleason et al., 2014).

4.2. Recovery of leaf-level transpiration and photosynthesis corresponds to the extent of embolism

The recovery of leaf physiological traits was influenced by the degree of embolism accumulation, reflecting variation in the sustained loss of hydraulic conductance. We did not observe a reversal of leaf vein emboli upon re-watering, which is consistent with previous findings (Cardoso et al., 2020; Johnson et al., 2018; Tonet et al., 2024). After re-watering, the function of recovered leaves was hindered, where average CO_2 assimilation and stomatal conductance were only ca. 60% of their initial, pre-stressed, values. This lasting depression of leaf-level traits could possibly be the result of compounding reductions in hydraulic conductivity in tissues upstream of leaves (e.g., root death, loss of capacitance tissues, stem embolism), although this hypothesis was not tested.

Plants were able to resume growth after watering regardless of the minimum leaf water potential they experienced during the dry down, indicating that points of growth within the stem were conserved during the dry down, avoiding desiccation (Bramley et al., 2015; Johnson et al., 2018). This spatial variation in sections of the plant that remained hydrated is also represented by water potential gradients between lower versus upper leaves (Figure 3.1; Tang and Boyer, 2008). This compartmentalized water loss likely aids plant resilience by placing meristematic and developing reproductive structures at a higher priority than leaves. Permitting seed development in the absence of photosynthesis by the movement of stored carbon or by aiding post-drought recovery and growth from the remobilization leaf compounds sourced from senescent lower leaves (Field, 1983; Jurgens et al., 1978). Observations of plant resilience amidst varying minimum leaf water potentials were consistent across all plants. Sustained leaf embolism and reduction in water transport capacity to leaves were a poor indicator of plant mortality, a trait that gives graminoid species a unique resilience in arid and semi-arid systems (Ocheltree et al., 2016). This resilience demonstrates the ability of maize to survive substantial drought periods while operating within the safety margin, with a long delay between stomatal closure and emboli formation, even when grown in pots.

4.3. Shrinkage

Here, we found that the rate of shrinkage accelerated rapidly after the onset of xylem embolism. This acceleration suggests that embolism obstruction upstream from the camera (measuring IVD) results in marked restriction of water delivery to the lamina and, thus, the relative water content of the lamina (Abate et al., 2021; Brodribb et al., 2021). However, this process was not entirely irreversible when the plants were re-watered (cf. Brodribb et al., 2021; Figure 5). Likewise, the onset of embolism did not always lead to the acceleration of leaf shrinkage, and the time between embolism onset and the acceleration of leaf shrinkage varied widely. The redundancy offered by parallel venation in maize, and more broadly, monocotyledon leaves might confer greater security from catastrophic consequences of embolism. For example, when a single vein is fully embolized, water can still be transported to distal portions of the leaf through other longitudinal and transverse veins. A stronger association between these events, such as the one reported by Brodribb et al. (2021), might exist in the leaves of dicotyledonous species that have a hierarchical venation pattern, where an embolism in one vein would block water transport to any distal portion of the leaf ‘downstream’ of that vein.

In the present study, leaf shrinkage provided a continuous, nondestructive estimate of internal water status from full hydration until complete desiccation and tissue death. Considering that pressure chamber measurements and stem psychrometers become increasingly difficult at low volumetric water contents (e.g., after the onset of embolism), leaf shrinkage may serve as a useful proxy for internal water status (Bourbia et al., 2023; Gleason et al., 2024).

4.4 Conclusions

Our findings show that maize can tolerate substantial embolism in developed leaves and that recovery after re-watering is aligned with the degree of embolism. Similar to a previous study on wheat, we did not observe embolism refilling in leaves after re-watering (Johnson et al., 2018). However, we note that both our study and the study by Johnson et al. (2018) used the leaf optical method, which in our view, is not well suited for detecting conduit refilling. The speed and nature of refilling in leaf veins (if it occurs) is not known, and we therefore do not know how it might appear in an image sequence, and the large differences in time and leaf area between full hydration and vein embolization make it difficult to compare image sequences. Variation in water loss rates among leaves at different positions highlights the dynamic nature of water regulation within the canopy of maize. Additionally, the prolonged depression of CO_2 assimilation in a laboratory setting may not directly apply to field conditions. The observed recovery of upper canopy leaves post-embolism emphasizes the importance of hydraulic resistance and compartmentalization in protecting sensitive tissues during desiccation, particularly in grasses that possess a well-defined culm. Prolonged dry down experiments of intact plants offer a more nuanced look into whole plant water dynamics and responses to severe water limitation. These insights enhance our understanding of plant-water relations and highlight the need to consider leaf shrinkage and whole plant dynamics in drought stress research, breeding programs, and water management practices.

CHAPTER 3: CONCLUSION AND FUTURE DIRECTIONS

This study examined the formation of xylem embolism in maize under controlled drought conditions, assessing its impact on both leaf-level physiology and whole-plant water utilization. Our findings emphasize the profound consequences of embolism on water transport, specifically its detrimental effect on the ability of leaves to sustain photosynthesis. In the previous chapter, accumulated embolism separated the magnitude of recovery of leaves. Without embolism, leaves recovered to pre-stress values by the end of the recovery period, while partially embolized leaves continued to have depressed values of photosynthesis and photosynthetic efficiency. This reinforces the idea that embolism limits water transport capacity, which hinders the recovery following drought but does not necessarily indicate leaf or plant mortality alone.

The compartmentalization of embolism to older and more mature leaves aligns with strategies observed in other grass species, where leaf or above-ground mortality during drought does not necessarily translate to whole-plant mortality. Embolism appeared to be constrained to afflicted leaf major veins, rarely spreading to other major veins after rewatering. In contrast to species with reticulate venation, such as dicotyledons, where high-order veins often embolize first impacting all tissues downstream, ultimately leading to leaf shedding (Cardoso et al., 2020; Hochberg et al., 2017). This is a potential point to investigate further as higher maize leaf orders would begin to desiccate from the tip towards the stem leaving variable amounts of seemingly functional leaf tissue near the ligule. It was unknown if embolism spread from the leaf tip. The initial loss of the lower leaves seemed more closely linked to leaf senescence processes, evident by the abrupt and coordinated declines of photosystem II efficiency and inter-vein distance around the time of embolism onset. Moreover, there was an apparent prioritization of developing leaves and reproductive structures over older, more mature leaves during drought stress. This was evident by water potential gradients between developing and mature leaves (Figure 3.1).

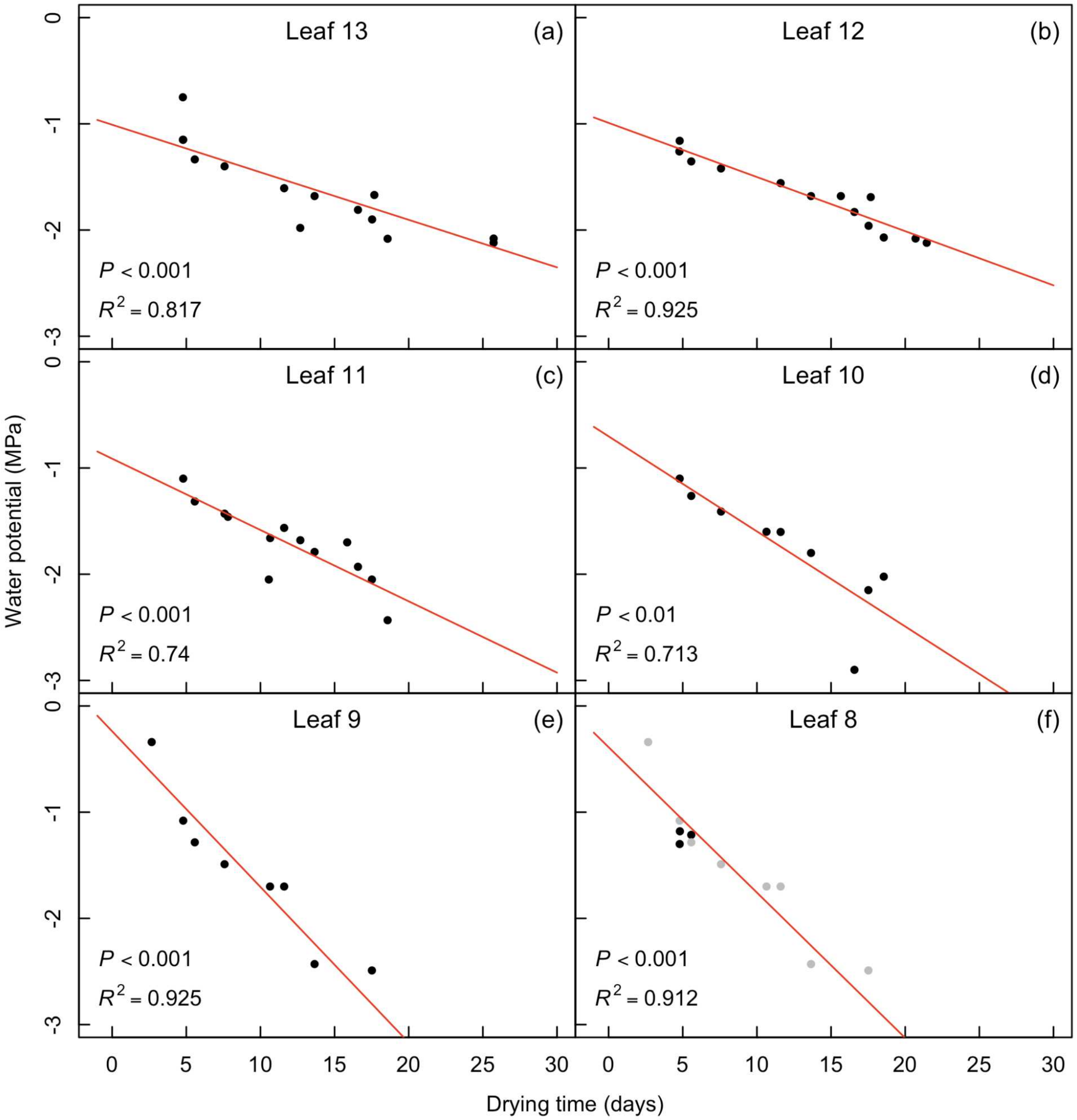

Figure 3.1. Measurements of leaf water potential (MPa) taken from companion plants during the dry down period. Measurements are from collared leaves numbered from the base. The 13th (a), 12th (b), 11th (c), 10th (d), 9th (e), and 8th, in black, pooled with leaf 9 measurements, in grey, for better predictions of water potential for this leaf (f). Fitted lines represent predicted water potentials used for individual leaves presented in Table 1. n = 19 (a), n = 15 (b), n = 15 (c), n = 11 (d), n = 11 (e), n = 11 (f) plants.

Developing leaves confined to the whorl maintained higher water potentials than their mature counterparts and were found to embolize later into the dry down. The proximity of developing leaves to the shoot apex gave a rough estimate of water potentials potentially experienced by the whorl. This suggests that maize, similar to other annual species, employs adaptive mechanisms to prioritize the development of reproductive structures in response to drought, ensuring seed set and population survival despite vegetative growth declines. This is consistent with observations of drought escape in annual plants, where reproductive success is achieved by allocating limited resources to developing tissues at the expense of vegetative tissues.

After rewatering, growth was observed to resume in the form of internode elongation and the expansion of leaves confined to the whorl. Despite resumed growth, there were sustained ill effects on whole plant water use, indicated by a 0.2 mmol s^{-1} reduction in whole plant transpiration compared to pre-stress values. This decrease is likely driven by the loss of functional leaf area by senescence and desiccation. This reduction of potential transpiration would likely impact potential yields as there is an established link between midday water use and end-of-season grain yields (Gleason et al., 2021). Future research should consider the onset of embolism and the potential for refilling in roots and stems, as these structures are critical for supporting leaf function. Additionally, exploring the role of stem water storage as a capacitive source, functioning to lengthen the time to hydraulic failure from the onset of drought conditions is needed to fully understand the large time gap (c.a. 15-20 days) from stomatal closure and embolization of the upper leaves observed in the controlled drought experiment (Gleason et al., 2014). how during drought would be crucial in understanding how maize and other grasses tolerate prolonged water deficits, and provide potential targets for crop improvement.

Another promising direction lies in identifying the genetic basis of embolism resistance and other drought tolerance traits in crop species. Identifying genotypic differences in hydraulic traits could uncover key genetic foundations related to xylem anatomy and hydraulic safety and efficiency, contributing greatly to drought-resilient crops (Gleason et al., 2022). These studies could extend beyond maize to other monocotyledon species, offering broader insights into drought responses in grasses, as vein and leaf structures are highly conserved within the Poaceae (Carlquist, 2012).

Leaf shrinkage, measured as inter-vein distance, emerged as a useful and non-destructive estimate of leaf water status in maize, with potential applications in agriculture and land management. Particularly for land management in semi-arid and arid systems where water availability fluctuates quickly. If leaf shrinkage measurements are effective and practical in field conditions, growers could use them to make informed irrigation decisions. This idea has been explored before with some success in citrus (Velez et al., 2007). Shrinkage traits may also serve as a general indicator of plant water status across different species, with the potential for strong associations with leaf embolism. This greatly increases the ease at which inferences of leaf embolism can be made in thin-leaved grass species where bundle sheath chloroplast obstructions often make the detection of embolism using the optical method difficult. Future studies should investigate the role of tissue shrinkage and water potential fluctuations in species with parallel venation, such as maize, to develop predictive models for drought stress and recovery.

Overall, the hydraulic system in plants plays a critical role in maintaining plant function under drought conditions. Processes such as photosynthesis and transpiration are sensitive to water availability, with embolism being the limiting factor for the recovery from drought stress. The lasting effects of embolism on plant functioning and the compartmentalization of damage to leaves suggest that dysfunction within the hydraulic pathway is a key determinant of drought-induced mortality. This study provides a foundation for future research to improve our understanding of whole-plant hydraulics, with implications for improving drought resilience in crop species through breeding and management practices.

In conclusion, the present study contributes to our understanding of drought responses in maize and offers insights into broader grassland systems. Embolism and leaf shrinkage are integrative traits that reflect complex physiological processes, such as water potential and tissue hydraulic capacity, making them valuable tools for predicting plant responses to water stress. Ongoing research into the mechanisms of embolism repair, water storage, and genetic variation in drought tolerance is crucial for enhancing crop resilience against extreme and unprecedented climate variability.

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